Studies on the Mechanism Regulating Reproductive Function in Puberty and the Application of Kisspeptin Analogs for the Treatment of Prostate Cancer

year	2020
その他のタイトル	性成熟期の生殖機能制御機構とキスペプチン誘導体の前立腺癌治療への応用に関する研究
学位授与大学	筑波大学 (University of Tsukuba)
学位授与年度	2019
報告番号	12102甲第9457号
URL	http://doi.org/10.15068/00160448

Studies on the Mechanism Regulating Reproductive Function in Puberty and the Application of Kisspeptin Analogs for the Treatment of Prostate Cancer

January 2020

Akira TANAKA

Studies on the Mechanism Regulating Reproductive Function in Puberty and the Application of Kisspeptin Analogs for the Treatment of Prostate Cancer

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biological Science
(Doctoral Program in Biological Sciences)

Akira TANAKA

Table of Contents

Abstract

Prostate cancer has high prevalence rate in men worldwide and there is no curable treatment established. Androgen deprivation therapy (ADT) is a gold standard therapy for advanced prostate cancer, however, eventually the cancer will be recurred by transforming to castration-resistant prostate cancer. Therefore, there is still an unmet medical need for a better treatment which can prolong or prevent the recurrence of prostate cancer. Thus, this study was aimed to develop a novel ADT agent which leads to better therapeutic outcome in the treatment of prostate cancer based on the better understanding of the mechanism regulating reproductive function in puberty.

In chapter 1, I studied on the mechanism regulating reproductive function in puberty focusing on how estrogen regulates gonadotropin releasing hormone (GnRH)/luteinizing hormone (LH) secretion in puberty in female rats. Reproductive function in mammalian species is centrally regulated by GnRH through the hypothalamus–pituitary–gonadal (HPG) axis. Puberty is a key process in the maturation of reproductive function in mammalian species, which can be defined as the initiation of GnRH/LH secretion. Previous study showed that estrogen strongly suppresses GnRH/LH secretion in prepubertal female rats, and its effectiveness is declined during pubertal period, however, the precise mechanism remained elusive. Kisspeptin is the endogenous ligand for human G-protein coupled receptor GPR54. With numerous efforts since two independent research groups reported that loss-of-function mutation of *GPR54* are responsible for pubertal failure in patients with hypogonadotropic hypogonadism, it has been suggested that kisspeptin-GPR54 signaling might be involved in the pubertal initiation of GnRH/LH secretion. In my study, I firstly identified that at least two distinct hypothalamic regions,

medial preoptic area (mPOA) and arcuate nucleus (ARC), are the action sites of estrogen for prepubertal restraint of GnRH/LH secretion in female rats in estradiol microimplant study in prepubertal and postpubertal ovariectomized (OVX) rats. I also determined that the attenuation of estrogen responsiveness observed in puberty is not due to the reduction of estrogen receptor expression in the mPOA and ARC in immunohistochemistry and gene expression analysis. Furthermore, I demonstrated that multiple rat kisspeptin analog rKp-10 or GnRH subcutaneous injections induce significant LH secretion in prepubertal OVX rats treated with systemic estradiol replacement. These findings suggest that a neural complex including estrogen-responsive neurons in the mPOA and ARC regulates GnRH/LH secretion in puberty possibly through modulating kisspeptin signals.

In chapter 2, I studied on the application of kisspeptin analogs for the treatment of prostate cancer. There are two different pharmacological approaches that can be considered to block kisspeptin-GPR54 signaling. One is the receptor antagonization and another is the receptor desensitization with the chronic treatment of agonist. Prior to my study, my team succeeded to synthesize Kiss-001 and Kiss-002 which are investigational kisspeptin analogs that have improved pharmacological stability with maintained potent agonistic activity for GPR54. In my study, I developed the one-month sustained release depot of Kiss-001 and Kiss-002, Kiss-001-SR(1M) and Kiss-002-SR(1M), and characterized their pharmacokinetics (PK), pharmacodynamics (PD) and efficacy profiles in animal models. Single subcutaneous injection of Kiss-001-SR(1M) and Kiss-002-SR(1M) maintained plasma Kiss-001 and Kiss-002 concentrations at certain levels for at least 4 weeks, before clearance from the circulation in intact male rats. Accompanying these PK profiles, Kiss-001-SR(1M) and Kiss-002-SR(1M) showed favorable PD responses. Both Kiss-001-SR(1M) and Kiss-002-SR(1M) demonstrated more rapid and

profound suppression of plasma testosterone levels than TAP-144-SR(1M), a GnRH agonist which is currently used in the treatment of prostate cancer, in intact male rats. These profound suppressive effects were maintained in dose-dependent manners, before recovery toward normal levels. In the JDCaP xenograft model, both Kiss-001-SR(1M) and Kiss-002-SR(1M) showed better prostate-specific antigen (PSA) control than TAP-144-SR(1M), although all treatment groups eventually experienced PSA recurrence and tumor regrowth. These study results indicate that both Kiss-001-SR(1M) and Kiss-002-SR(1M) have desirable and better PK/PD profiles than TAP-144-SR(1M) in rats, which could potentially provide better clinical outcomes in the treatment of androgen-dependent prostate cancer.

In conclusion, my study demonstrates that estrogen-responsive neurons located in the mPOA and ARC play important role in estrogen-dependent pubertal change of GnRH/LH secretion in female rats, and contributes better understanding on the mechanism regulating reproductive function in puberty. Also, my study indicates that kisspeptin analogs are applicable for the treatment of prostate cancer and Kiss-001-SR(1M) and Kiss-002-SR(1M) are the promising clinical candidates to be tested in patients with prostate cancer.

Abbreviations

ADPC	androgen dependent prostate cancer
ADT	androgen deprivation therapy
ARC	arcuate nucleus
CRPC	castration resistant prostate cancer
ER	estrogen receptor
FSH	follicular stimulating hormone
GnRH	gonadotropin releasing hormone
HPG	hypothalamus-pituitary-gonadal
Kiss-001F	Free form of Kiss-001
Kiss-002F	Free form of Kiss-002
LH	luteinizing hormone
LLOQ	lower limit of quantification
mPOA	medial preoptic area
mRNA	messenger ribonucleic acid
ORX	orchiectomy
OVX	ovariectomy
PD	pharmacodynamics
PK	pharmacokinetics
PSA	prostate specific antigen
PVN	periventricular nucleus
VMH	ventromedial hypothalamus

General introduction

Prostate cancer is the second most frequent cancer diagnosis made in men and the fifth leading cause of death worldwide (Prashanth, 2019). Prostate cancer is generally found in elderly man and there is no curable treatment developed so far. In the most case, prostate cancer repeats the recurrence (Fig. 1). Therefore, sequential treatment has been developed (Mohler et al., 2019).

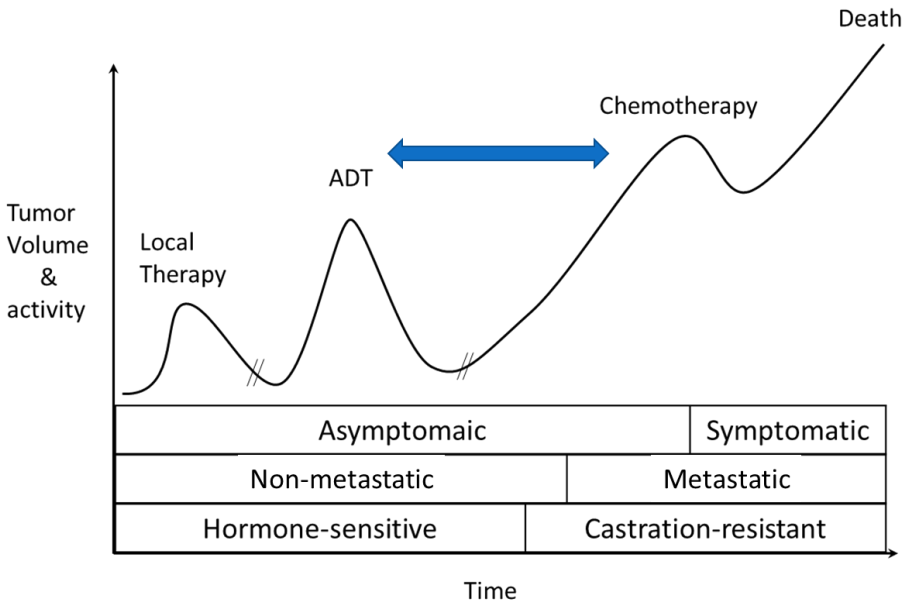

Fig. 1 The schematic illustration of natural history of prostate cancer and sequential treatment. Prostate cancer at early stage has asymptomatic, non-metastatic and hormone-sensitive characteristics. Then, it transforms to symptomatic, metastatic and castration-resistant cancer after repeating the recurrence. ADT: Androgen deprivation Therapy.

Prostate cancer can be found at relatively early stage regardless its asymptomatic and slow growth because there is a highly sensitive screening tool measuring prostate specific antigen (PSA) in blood. The standard treatment for early stage prostate cancer is watchful waiting or local therapy such as prostatectomy or radiation. Next sequential treatment is androgen deprivation therapy (ADT) because prostate cancer primarily grows

in an androgen dependent manner. There are various ADT regimens available such as bilateral orchiectomy (ORX), a gonadotropin releasing hormone (GnRH) agonist or antagonist, or a GnRH agonist plus an anti- androgen. ADT is an effective treatment for castration-naïve prostate cancer, however, after a certain period, most prostate cancer eventually recurs by transforming to castration-resistant prostate cancer (CRPC). Therefore, there is still an unmet medical need for a better treatment which can prolong or prevent the recurrence of prostate cancer.

Reproductive function in mammalian species is centrally regulated by GnRH through the hypothalamus–pituitary–gonadal (HPG) axis (Fig. 2). GnRH released from the hypothalamus stimulates the pituitary gland to secrete gonadotropins, luteinizing hormone (LH) and follicular-stimulating hormone (FSH). Either of LH and FSH plays an important role in gonadal function which is the synthesis of gonadal steroids such as testosterone and estradiol, spermatogenesis in testes or oocyte maturation in ovaries. Then, estradiol feedback to the hypothalamus to maintain the homeostasis by regulating GnRH releasing activity. In addition to this feedback loop, there is a female specific feedback system named positive feedback which triggers the ovulation. It is still unknown how estradiol regulates GnRH neuronal activity in both feedback systems because GnRH neuron does not have estrogen receptor (ER). It is assumed that estrogen responsive neurons in hypothalamus may intermediate the feedback signals to GnRH neurons.

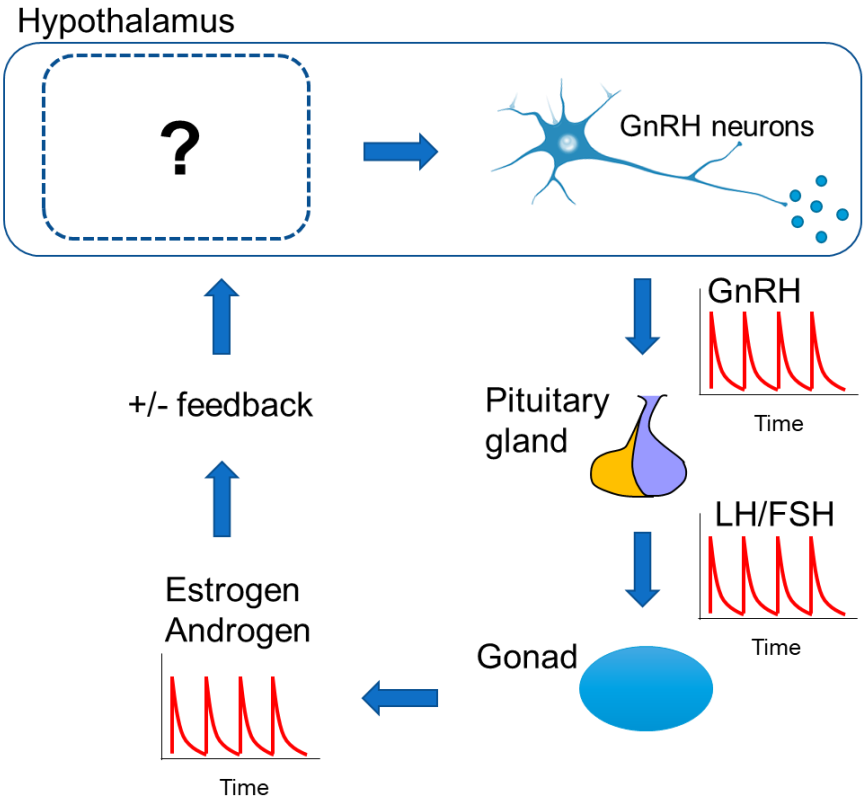

Fig 2. Regulating mechanism of reproductive function in mammalian species. Reproductive function in mammalian species is centrally regulated by GnRH through the HPG axis. GnRH released from the hypothalamus stimulates the pituitary gland to secrete gonadotropins, LH and FSH. Either of LH and FSH plays an important role in gonadal function which is the synthesis of gonadal steroids such as testosterone and estradiol, spermatogenesis in testes or oocyte maturation in ovaries. Then, estradiol feedback to the hypothalamus to maintain the homeostasis by regulating GnRH releasing activity. In addition to this feedback loop, there is a female specific feedback system named positive feedback which triggers the ovulation.

Puberty is a key event in the maturation process of reproductive function in mammalian species. Puberty can be defined as the activation of HPG axis, which is equal to the initiation of GnRH/LH secretion (Fig. 3). Previous study showed that estrogen strongly suppresses GnRH/LH secretion in prepubertal female rats and its effectiveness is declined during pubertal period (Andrews et al., 1981), however, the precise mechanism remained elusive.

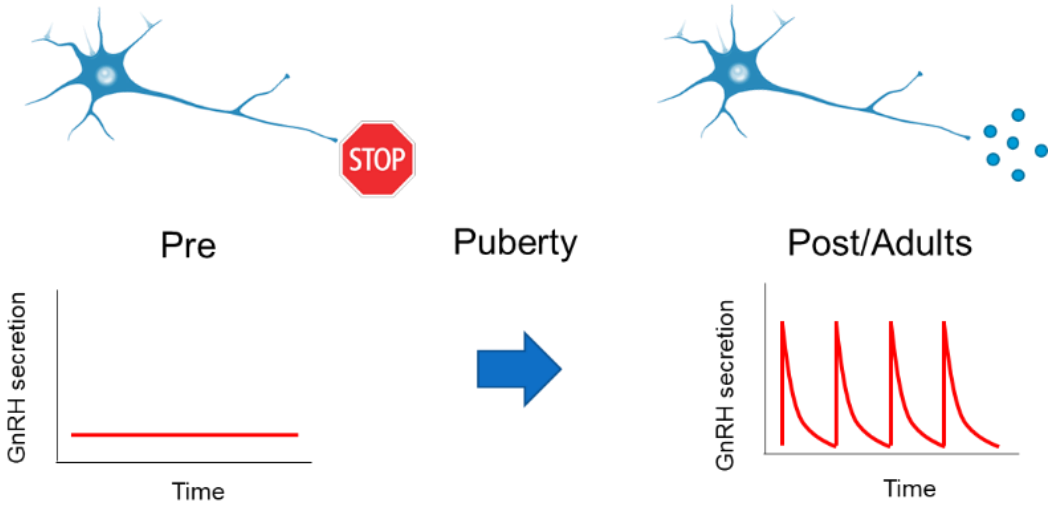

Fig. 3 Schematic illustration of pubertal transition of GnRH/LH secretion. Puberty can be defined as the initiation of GnRH/LH secretion.

Kisspeptin is a protein encoded by the *KISS1* gene. Kisspeptin is also known as metastin because *KISS1* gene was originally identified as a tumor metastasis suppressor gene which can suppress the metastasis of melanoma and breast cancer (Jeong-Hyung et al, 1996). Kisspeptin is the endogenous ligand for human G-protein coupled receptor GPR54 (Ohtaki et al., 2001). In 2003, two independent groups reported that loss-of-function mutation of *GPR54* are responsible for pubertal failure in patients with hypogonadotropic hypogonadism (de Roux et al., 2003; Seminara et al., 2003). In addition to these findings, loss of function mutation of *KISS1* is also identified in patients with hypogonadotropic hypogonadism (Topaloglu et al., 2012). These phenotypes were reproduced in *Kiss1* or *Gpr54* knockout mice (Seminara et al., 2003; Messager et al., 2005; d'Anglemont et al., 2007; Dungan et al., 2007; Lapatto et al., 2007; Chan et al., 2009). These findings suggest that kisspeptin-GPR54 signaling could be involved in the pubertal initiation of GnRH/LH secretion.

There are two different pharmacological approaches that can be considered to block kisspeptin-GPR54 signaling. One is the antagonizing the receptor function (Kobayashi et al., 2010; Roseweir et al., 2013). Another is the desensitizing the receptor with chronic treatment of agonist (Rajagopal et al., 2018). Indeed, it was demonstrated that chronic administration of kisspeptin resulted in the suppression of reproductive function in adult male rats (Thompson et al., 2006) and agonadal juvenile male monkeys (Seminara et al., 2006; Ramaswamy et al., 2007). Thus, my team developed Kiss-001 (another name: TAK-448) and Kiss-002 (another name: TAK-683) which are therapeutic kisspeptin analogs that have improved pharmacological stability with maintained potent agonistic activity for GPR54 compared with the natural peptide (Nishizawa et al., 2016).

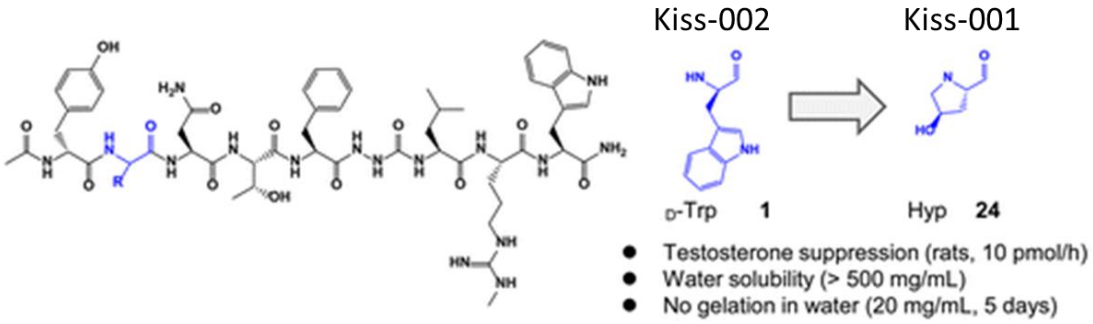

Fig. 4. Kisspeptin agonists, Kiss-001 and Kiss-002 (modified from Nishizawa et al., J Med Chem 2017). Kiss-001 and Kiss-002 are kisspeptin analogues with trans-4-hydroxyproline (Hyp) and D-tryptophan (D-Trp) at position 47, respectively.

This study was aimed to develop a novel ADT agent which lead to better therapeutic outcome in the treatment of prostate cancer based on the better understanding of the mechanism regulating reproductive function including androgen synthesis in puberty. In chapter 1, I studied on the mechanism regulating reproductive function in puberty focusing on how estrogen regulates GnRH/LH secretion in puberty in female rats. I determined which central action site(s) of estrogen is involved in the prepubertal restraint

of GnRH/LH secretion and clarified the pubertal changes in response to estrogen action in female rats. Also, I examined the effect of exogenous kisspeptin on prepubertal GnRH/LH secretion to understand on the mechanism regulating GnRH/LH secretion in puberty. In chapter 2, I studied on the application of kisspeptin analogs for the treatment of prostate cancer. I determined the pharmacokinetics (PK) and pharmacodynamics (PD) of Kiss-001-SR(1M) and Kiss-002-SR(1M), one-month sustained release depot of Kiss-001 and Kiss-002, in intact male rats. Furthermore, I investigated the anti-tumor effect of Kiss-001-SR(1M) and Kiss-002-SR(1M) in the JDCaP xenograft rat model to discuss their therapeutic advantages over TAP-144-SR(1M).

Chapter 1: Central Estrogen Action Sites Involved in Prepubertal Restraint of Pulsatile Luteinizing Hormone Release in Female Rats

Abstract

The present study aimed to determine estrogen feedback action sites to mediate prepubertal restraint of gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) release in female rats. Wistar-Imamichi strain rats were ovariectomized (OVX) and received a local estradiol-17β (estradiol) or cholesterol microimplant in several brain areas, such as the medial preoptic area (mPOA), paraventricular nucleus, ventromedial nucleus and arcuate nucleus (ARC), at 20 or 35 days of age. Six days after receiving the estradiol microimplant, animals were bled to detect LH pulses at 26 or 41 days of age, representing the pre- or postpubertal period, respectively. Estradiol microimplants in the mPOA or ARC, but not in other brain regions, suppressed LH pulses in prepubertal OVX rats. Apparent LH pulses were found in the postpubertal period in all animals bearing estradiol or cholesterol implants. It is unlikely that pubertal changes in responsiveness to estrogen are due to a change in estrogen receptor (ER) expression, because the number of ERα-immunoreactive cells and mRNA levels of *Esr1*, *Esr2* and *Gpr30* in the mPOA and ARC were comparable between the pre- and postpubertal periods. In addition, kisspeptin or GnRH injection overrode estradiol-dependent prepubertal LH suppression, suggesting that estrogen inhibits the kisspeptin-GnRH cascade during the prepubertal period. Thus, estrogen-responsive neurons located in the mPOA and ARC may play key roles in estrogen-dependent prepubertal restraint of GnRH/LH secretion in female rats.

Introduction

In mammals, sexual maturation at puberty onset seems to be timed by an increase in pulsatile gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) secretion (Andrew et al., 1981; Foster et al., 1985; Foster et al., 1988; Watanabe et al., 1989; Sisk et al., 2001). Previous studies showed that prepubertal restraint of GnRH/LH secretion seems to be controlled by gonad-dependent and gonad-independent mechanisms according to the species: plasma LH levels increased immediately after ovariectomy in prepubertal sheep (Foster et al., 1988) and rats (Andrew et al., 1981), whereas plasma LH levels remained low even after ovariectomy in prepubertal rhesus monkeys (Chongthammakun et al., 1993; Pohl et al., 1995). The precise mechanisms regulating the prepubertal restraint of and pubertal increase in GnRH/LH secretion remain very elusive. To date, the most plausible explanation has been that kisspeptin-GPR54 signaling controls the pubertal increase in GnRH/LH release, because loss-of-function mutations of *KISS1* or *GPR54* are responsible for pubertal failure in patients with hypogonadotropic hypogonadism (de Roux et al., 1995; Seminara et al., 2003; Topaloglu et al., 2012). These phenotypes were duplicated in *Kiss1* or *Gpr54* knockout mice (Seminara et al., 2003; Messager et al., 2005; d'Anglemont et al., 2007; Dungan et al., 2007; Lapatto et al., 2007; Chan et al., 2009). Indeed, *Kiss1* gene expression in the anteroventral periventricular nucleus and hypothalamic arcuate nucleus (ARC) in rodents is nearly absent in the prepubertal period and increases at the onset of puberty (Navarro et al., 2004; Clarkson et al., 2006; Takase et al., 2009). Since ARC *Kiss1* mRNA expression is strongly suppressed by estradiol-17β (estradiol) derived from immature ovaries in prepubertal female rats (Takase et al., 2009), ARC kisspeptin neurons are assumed to be a target of

estrogen negative feedback action, which restrains GnRH/LH secretion in rodents during the prepubertal period.

The estrogen receptor α (ERα) seems to play a critical role in estrogen feedback action, because ERα knockout mice showed constant high plasma LH levels regardless of estrogen treatment, indicating a lack of estrogen-negative and estrogen-positive feedback actions (Couse et al., 1999). It was shown that tritiated estradiol did not concentrate in GnRH neurons in adult rats (Shivers et al, 1983). In addition, immunohistochemistry for ERα and in situ hybridization analysis for *Esr1* mRNA encoding ERα revealed the absence of ERα expression in rodent GnRH neurons (Herbison et al, 1992; Herbison et al., 2001). Thus, non-GnRH neurons expressing ERα, such as kisspeptin neurons (Kinoshita et al., 2005; Smith et al., 2005; Adachi et al., 2007), may play a critical role in prepubertal restraint of GnRH/LH secretion. In the rodent brain, ERα or *Esr1* mRNA expression is abundantly found in discrete areas of the hypothalamus, such as the medial preoptic area (mPOA), ventromedial nucleus (VMH) and ARC. In addition, de novo ERα expression in the paraventricular nucleus (PVN) and nucleus of the solitary tract was reported to be involved in fasting-induced LH suppression in rats (Nagatani et al., 1994; Estacio et al., 1996). Nevertheless, the action site(s) of estrogen that suppresses GnRH/LH secretion during the prepubertal period remains poorly understood. Determination of the precise action site(s) of estrogen should provide clues for identifying target neurons involved in the estrogen-dependent restraint of GnRH/LH release in prepubertal females.

The present study aimed to determine which central action site(s) of estrogen is involved in the prepubertal restraint of GnRH/LH release and to clarify the pubertal changes in response to estrogen action in female rats. To this end, I examined if placement

of estradiol microimplants into discrete brain areas, such as the mPOA, PVN, VMH and ARC, causes suppression of pulsatile LH secretion in OVX prepubertal and postpubertal female rats. I also determined the expression of ERs in the mPOA and ARC, because I found that estradiol microimplants in these two nuclei inhibited pulsatile LH release in prepubertal OVX animals.

Materials and Methods

Animals

Wistar-Imamichi strain rats were kept under a 14:10 h light/dark cycle (lights on at 05:00 h) at 22 ± 2℃ with free access to food (CE-2; CLEA Japan, Tokyo, Japan) and water. Female rats (8-10 weeks old of age) having at least two consecutive regular 4-day estrus cycles were mated with males overnight on the day of proestrus, and then the pregnant females were housed individually. The day on which a newborn litter was found at noon was designated postnatal day 0. The litter size was adjusted to eight on day 1 to minimize the growth variation within and between litters. The pups were weaned on day 20. Vaginal openings were checked every morning thereafter. After vaginal opening, vaginal smears were taken daily to determine the day of first estrus and estrous cyclicity. Since a preliminary study in female rats showed vaginal opening and first estrus at 29.6 ± 0.3 and 34.6 ± 0.9 days of age, respectively, days 26 and 41 were designated representative days for the pre- and postpubertal periods. Surgical procedures were performed under anesthesia with a ketamine-xylazine mixture.

Care of the animals and all of the experimental procedures used in these experiments were approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences, Nagoya University (Permit No. 2012031616).

Intracranial or subcutaneous estradiol implant

Rats were ovariectomized (OVX), and some of them received unilateral estradiol (Sigma-Aldrich, St. Louis, MO, USA) or cholesterol microimplants in the various hypothalamic regions in the pre- (day 20 of age) or postpubertal period (day 35 of age). The microimplants were made of a 1:1000 (w/w) mixture of estradiol and paraffin.

Cholesterol was used instead of estradiol in controls. The paraffin mixture (approximately 0.05 mm^3), theoretically containing 33 ng estradiol or cholesterol, was then punched out with a 24-gauge stainless steel tubing (0.30 mm i.d.). The stainless steel tubing containing the paraffin mixture was then stereotaxically inserted in the discrete area of the hypothalamus. The paraffin mixture was immediately pushed out of the tubing and into the designated nucleus using a stainless steel wire (0.25 mm diameter). The stereotaxic coordinates (mm posterior and ventral to the bregma, and lateral to the midline) were as follows: mPOA, 0.8, 7.7, 0.4; PVN, 1.8, 7.4, 0.5; VMH, 2.8, 9.0, 0.8; and ARC, 2.8, 9.0, 0.5.

Some of the OVX animals were subcutaneously implanted with a Silastic tubing (1.57 mm i.d.; 3.18 mm o.d.; 8 mm in length for day 20 and 16 mm in length for day 35, Dow Corning, Midland, MI, USA) containing estradiol dissolved in peanut oil at 20 μg/ml to mimic the diestrous plasma estradiol level in ovary-intact female rats (Takase et al., 2009; Cagampang et al., 1991).

Blood sampling

On days 26 or 41 of age, i.e., 6 days after the estradiol treatment in the pre- or postpubertal groups, blood samples (50 μl) were collected from the free-moving animals for 3 h at 6-min intervals, starting at 13:00, through a silicone catheter (0.5 mm i.d., 1.0 mm o.d., Shin-Etsu Polymer, Tokyo, Japan) inserted into the right atrium through the jugular vein on the day before blood sampling. An equivalent volume of rat red blood cells, taken from donor rats and diluted with heparinized saline, was replaced through the cannula after each blood collection to keep the hematocrit constant. Plasma was separated by immediate centrifugation and stored at -20℃ until assayed for LH.

Brain histology

After blood sampling, animals bearing the intracranial microimplants were perfused with saline followed by 10% formalin under deep anesthesia with sodium pentobarbital to confirm the placement of the microimplants. Coronal sections of the brain made with a cryostat were stained with thionin, and the sites of the microimplants were verified under a microscope according to a rat brain atlas (Paxinos et al., 2007). Some animals bearing microimplants in an undesignated nucleus were eliminated from the further analysis.

Uteri collection to verify systemic leakage of estradiol

Uteri were collected immediately before perfusion and weighed. The wet weights of the uteri were not significantly different between intracranial estradiol- and cholesterol-implanted animals (data not shown), indicating no systemic leakage of estradiol from the intracranial microimplants.

Effect of intravenous kisspeptin or GnRH challenge on LH release in prepubertal rats

To test if the estrogen-dependent prepubertal suppression of LH release is due to the restraint of GnRH and/or kisspeptin release, subcutaneous estradiol-treated OVX rats at 26 days of age received four intravenous injections of the C-terminal decapeptide of rat kisspeptin (rKp-10, 10 nmol/kg body weight, n=3) or GnRH (0.1 nmol/kg, n=3) at 1, 1.5, 2 and 2.5 h after the onset of blood sampling. Blood samples (50 μl) were obtained every 6 min for 3 h (13:00-16:00).

LH assay

Plasma LH concentrations were determined by a double-antibody radioimmunoassay (RIA) using a rat LH RIA kit provided by the National Hormone and Peptide Program (Torrance, CA, USA) and were expressed in terms of the NIDDK rat LH-RP-3. The least detectable level was 7.8 pg/tube for 25 μl plasma samples. Intra- and inter-assay coefficients of variation were 8.0% and 13.8% at the level of 0.78 ng/ml, respectively.

Brain tissue sampling and immunohistochemistry

At 26 or 41 days of age, animals were deeply anesthetized with pentobarbital and then perfused with phosphate-buffered saline followed by 4% paraformaldehyde. Serial frontal sections containing the entire mPOA or ARC (30 μm) were made on a cryostat, and every 8th section was immunostained with anti-ERα antibody (Upstate Biotechnology, Lake Placid, NY, USA) as previously described (Yamada et al., 2006). Briefly, brain tissue sections were incubated with the anti-ERα antibody (1:40,000) for 4 days at 4℃, followed by incubation in biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories, Burlingame, CA, USA) for 90 min and avidin biotin complex for 60 min. ERα immunoreactivities were visualized using 3,3'-diaminobenzidine as a chromogen.

Brain tissue sampling and quantitative RT-PCR for gene expression of Esr1 (ERα), Esr2 (ERβ) and Gpr30 (membrane estrogen receptor) in the mPOA and ARC

At 26 or 41 days of age, brain tissues were obtained from female rats after decapitation between 13:00 and 14:00. Since the present results suggested that the mPOA

and ARC were estrogen action sites for prepubertal restraint of LH pulses, tissue samples containing the mPOA or ARC were obtained from 2-mm-thick brain tissue slices according to a rat brain atlas (Paxinos et al., 2007). The anterior and posterior ends of tissue slices for the mPOA were approximately 0.48 mm anterior and 1.44 mm posterior to the bregma, respectively. The mPOA region was punched out with 18-gauge stainless steel tubing. The anterior and posterior ends of tissue slices for the ARC were approximately 1.80 mm and 4.20 mm posterior to the bregma, respectively. The ARC-median eminence (ARC-ME) region was dissected out with a microknife. Fifty-micrometer-thick coronal sections were made from the remaining brain tissues and stained with thionin to verify the brain regions dissected or punched out under a microscope according to a rat brain atlas (Paxinos et al., 2007).

Expression of *Esr1*, *Esr2* and *Gpr30* mRNA was determined by quantitative RT-PCR in the mPOA and ARC-ME regions. Real-time RT-PCR analysis was performed using an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Briefly, DNA-free total RNA was extracted from the tissues using ISOGEN (Nippon Gene, Tokyo, Japan) and DNase I (Invitrogen, Carlsbad, CA, USA). cDNA from each RNA sample was synthesized with a random primer using a High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. The following cycling protocol was used: 2 min at 50℃, 10 min at 95℃ and 40 cycles amplification for 15 sec at 95℃ and 1 min at 60℃. Predesigned primers and probes (TaqMan Gene Expression Assay, IDs: Rn00562166_m1 for *Esr1*, Rn00562610_m1 for *Esr2*, Rn005920911_s1 for *Gpr30* and Rn00667869_m1 for *Actb*) were purchased from Applied Biosystems.

Data analysis and statistical analysis

LH pulses were determined by the PULSAR computer program (Merriam et al., 1982). Each individual's mean LH concentrations and frequency and amplitude of LH pulses were calculated for the 3-h sampling period. Statistical differences in the LH pulse parameters and uterine weight were determined by a two-way (age and estradiol treatment as main effects) ANOVA (R version 3.1.1, http://www.R-project.org/). If the interaction was significant, simple main effects were determined.

Changes in the LH pulse frequency in each individual with an estradiol microimplant were calculated by subtracting the mean frequency of cholesterol-implanted controls from the frequency of each individual with an estradiol implant within each hypothalamic area.

The obvious ERα-immunoreactive cells in the mPOA and ARC and on their border were unilaterally counted twice by one of the authors under a microscope, and the average was calculated. The areas examined in the present study were identified based on a rat brain atlas (Paxinos et al., 2007). The total numbers of sections used for quantification of ERα-immunoreactive cells were 4 and 8 for the mPOA and ARC, respectively.

The copy numbers of *Esr1*, *Esr2* and *Gpr30* transcripts were normalized to the ratio of the copy number of *Actb* transcripts for each sample. Statistical differences between age were determined by Student's *t*-test. Values were considered significant when $P < 0.05$.

Results

Placement of estradiol microimplants and their effects on LH pulse frequency in individuals

Figure 5 summarizes the placement of estradiol microimplants in the mPOA, ARC, PVN and VMH and changes in the number of LH pulses in each individual compared with the mean LH pulse frequency in the cholesterol-implanted controls. LH pulse frequency was decreased in all individuals with estradiol implants placed in the mPOA or ARC at 26 days of age compared with the cholesterol-implanted controls. On the other hand, no apparent change was found in animals with estradiol implant in the mPOA or ARC at 41 days of age or in those with estradiol implant in the PVN or VMH at 26 and 41 days of age. Cholesterol microimplants were placed in similar locations in each group (data not shown).

Effects of estradiol microimplants on pulsatile LH release in pre- and postpubertal OVX rats

Plasma LH profiles of representative OVX rats bearing estradiol or cholesterol implants are shown in Fig. 6. Regular LH pulses were found in rats with cholesterol microimplants in any of the hypothalamic regions at both 26 and 41 days of age. Estradiol microimplants into the mPOA or ARC caused partial suppression of pulsatile LH secretion at day 26. On the other hand, estradiol microimplants in the PVN or VMH had no apparent effect on LH secretion at 26 days of age. Pulsatile LH secretion was strongly suppressed at day 26 in OVX females with subcutaneous estradiol treatment. Regular LH pulses were found in OVX rats with estradiol microimplants in any of these hypothalamic

regions or with subcutaneous treatment at 41 days of age.

Effects of estradiol microimplants on LH pulse parameters in pre- and postpubertal OVX rats

Three LH pulse parameters, i.e., mean LH concentration (Fig. 7A), frequency (Fig. 7B) and amplitude (Fig. 7C) of LH pulses, in pre- and postpubertal OVX rats treated with estradiol or cholesterol are shown in Fig. 7.

The estradiol microimplants in the mPOA suppressed pulsatile LH secretion only in the prepubertal period. Specifically, two-way ANOVA revealed that estradiol microimplants into the mPOA (main effect) significantly suppressed LH pulse frequency ($F(1,17) = 11.9$; *, $P < 0.05$), and the main effect was qualified by significant interaction ($F(1,17) = 7.1$, $P < 0.05$). More specifically, estradiol-dependent suppression of LH pulse frequency was significant in the prepubertal period (†, $P < 0.05$, Fig. 7B), but not in the postpubertal period. Further, the frequency of LH pulses was lower in the prepubertal period than in the postpubertal period in rats bearing estradiol microimplants in the mPOA (§, $P < 0.05$, Fig 7B). Also, estradiol microimplants in the mPOA failed to significantly change the mean LH concentrations and amplitude of LH pulses between the pre- and postpubertal periods.

The estradiol microimplants in the ARC suppressed pulsatile LH secretion in both the pre- and postpubertal periods. Specifically, two-way ANOVA revealed that ARC estradiol microimplants (main effect) significantly suppressed both mean LH concentrations ($F(1,14) = 4.7$, *, $P < 0.05$, Fig. 3A) and LH pulse frequency ($F(1,14) = 12.3$, *, $P < 0.05$, Fig. 3B). Further statistical analyses were omitted because the main effect of estradiol microimplants in the ARC on mean LH concentration or LH pulse

frequency was not qualified by significant interaction.

On the other hand, the estradiol microimplants into the PVN or VMH failed to suppress pulsatile LH secretion in both the pre- and postpubertal periods, because no significant change was found in any pulse parameters in rats with PVN or VMH estradiol microimplants (Fig. 7).

The subcutaneous estradiol treatment suppressed pulsatile LH secretion in both the pre- and postpubertal periods. Specifically, two-way ANOVA revealed that subcutaneous estradiol treatment (main effect) significantly suppressed three LH pulse parameters (mean LH, $F(1,16) = 60.4$, *, $P < 0.05$, Fig. 7A; frequency, $F(1,16) = 45.6$, *, $P < 0.05$, Fig. 7B; amplitude, $F(1,15) = 6.2$, *, $P < 0.05$, Fig. 7C). The main effects of estradiol replacement on mean LH concentration and LH pulse frequency were qualified by significant interactions (mean LH, $F(1,16) = 7.3$, $P < 0.05$; frequency, $F(1,16) = 14.7$, $P < 0.05$). More specifically, subcutaneous estradiol treatment significantly suppressed mean LH concentrations in both the pre- and postpubertal periods (†, $P < 0.05$, Fig. 7A). Estradiol-dependent suppression of LH pulse frequency was found in the prepubertal period (†, $P < 0.05$, Fig. 7B), but not in the postpubertal period. The frequency of LH pulses was significantly lower in the prepubertal period than that in the postpubertal period in OVX rats with subcutaneous estradiol treatment (§, $P < 0.05$, Fig. 7B).

Expression of ERs in the mPOA and ARC of pre- and postpubertal OVX rats

A large number of ERα-immunoreactive cells were found at 26 and 41 days of age in the mPOA and ARC in pre- and postpubertal OVX rats (Fig. 8A). In both nuclei, no significant difference was found in the number of ERα-immunoreactive cells between 26 and 41 days of age (Fig. 8B). *Esr1, Esr2 and Gpr30* mRNA levels were quantitated in the

mPOA and ARC-ME tissues, which are schematically illustrated as shaded areas in Figure 5A. Dissected mPOA tissues included most of the medial preoptic nucleus and a part of the ventral limb of the diagonal band and median preoptic nucleus. Dissected ARC tissues included most of the ARC-ME region, a small portion of the VMH and the anterior part of the mammillary body nucleus. No significant difference in the expression of *Esr1*, *Esr2* and *Gpr30* was found between 26 and 41 days of age (Fig. 9B). The expression of *Esr1* was relatively higher than that of *Esr2* and *Gpr30* in both nuclei (Fig. 9B).

Effect of intravenous rKp-10 or GnRH challenge on LH release in prepubertal OVX rats subcutaneously treated with estradiol

Figure 10 shows the effects of rKp-10 or GnRH challenge on plasma LH levels in representative prepubertal (day 26) OVX rats with subcutaneous estradiol treatment. Four consecutive injections of rKp-10 or GnRH immediately increased plasma LH levels, while plasma LH levels remained low throughout the sampling period in the saline-injected controls. Every intravenous injection of rKp-10 or GnRH acutely increased the plasma LH level, which peaked within 12 min after injection and then declined to the basal level by 30 min after injection, mimicking spontaneous LH pulses in postpubertal rats.

Discussion

The present study suggests that at least two distinct hypothalamic regions, i.e., the mPOA and ARC, are action sites of estrogen for prepubertal restraint of LH release, because estradiol microimplants in the mPOA or ARC were capable of suppressing LH pulses in prepubertal OVX female rats. It also suggests that it is unlikely that estradiol implanted in the mPOA and ARC leaks into the circulation and acts on other sites, because the uterine weight of the rats with estradiol microimplants was comparable to that of cholesterol-treated controls. Indeed, estradiol microimplants in the PVN or VMH showed no suppressive effect on LH pulses in OVX rats in the prepubertal stage, suggesting that the effect of estradiol microimplants was largely limited within the precise brain area and that the mPOA and ARC are the estrogen action sites involved in the prepubertal suppression of LH pulses. Thus, in intact prepubertal female rats, circulating estrogens derived from the premature ovary may act on the mPOA and ARC, leading to the prepubertal restraint of GnRH/LH release. In addition, kisspeptin or GnRH challenge in the present study successfully stimulated LH secretion in prepubertal OVX rats with subcutaneous estradiol treatment, indicating that estrogen-dependent prepubertal restraint of LH release would be due to the suppression of kisspeptin and subsequent GnRH release. This notion is consistent with previous studies, which showed that changes in ARC *Kiss1*/kisspeptin expression are consistent with those in LH pulses in the peripubertal period and suggested that prepubertal LH suppression is mainly due to the suppression of kisspeptin expression/release and subsequently GnRH release (Navarro et al., 2004; Takase et al., 2009).

The present study also suggests a pubertal decrease in the responsiveness to

estrogen in the mPOA, because mPOA estradiol microimplants failed to cause obvious suppression of LH pulse frequency in postpubertal animals. On the other hand, estradiol microimplants in the ARC constantly suppressed mean LH concentrations and LH pulse frequency in both the pre- and postpubertal periods. These results suggest that estrogen-responsive neurons in the mPOA and ARC play key roles in pubertal restraint of GnRH/LH secretion, whereas the ARC estrogen-responsive neurons mediate the negative feedback action of estrogen on GnRH/LH secretion in the postpubertal period. The current study showed that the number of ERα-expressing cells and the *Esr1*, *Esr2* or *Gpr30* expression in the mPOA and ARC were comparable between the pre- and postpubertal periods. Thus, it is unlikely that changes in responsiveness to estrogen negative feedback action during puberty on GnRH/LH suppression is simply caused by a change in expression of these ERs in the mPOA. It is possible that increases in stimulatory inputs or decreases in inhibitory inputs from the mPOA estrogen-responsive neurons to the GnRH pulse generator rather than a change in the expression of ERs would be involved in the different responses to estrogen between pre- and postpubertal female rats. Further, the current results obtained from OVX rats with subcutaneous estradiol treatment seem to be a summation of those obtained from the animals with mPOA or ARC estradiol microimplants in terms of two out of three LH pulse parameters (mean LH concentrations and LH pulse frequency). It cannot be ruled out that estrogen-responsive neurons in brain areas other than the mPOA and ARC are involved in the prepubertal restraint of LH secretion, because subcutaneous estradiol treatment profoundly suppressed LH pulses during the prepubertal period. Detailed analysis is needed to clarify these issues in the future.

The present study suggests that estrogen-responsive neurons located in the mPOA

and ARC may play critical roles in the prepubertal restraint of kisspeptin and GnRH/LH secretion. The most plausible explanation for the current results using ARC estradiol microimplants is that ARC kisspeptin neurons could well serve as direct targets for estrogen negative feedback action for prepubertal restraint of GnRH/LH release in female rats. Indeed, ARC kisspeptin neurons exhibit ERα expression (Kinoshita et al., 2005; Smith et al., 2005; Adachi et al., 2007), and ARC *Kiss1* gene expression is nearly absent in the prepubertal period and increases at the onset of puberty (Takase et al., 2009). In addition, ARC *Kiss1* expression was enhanced after ovariectomy and was strongly suppressed by estradiol replacement in prepubertal female rats (Takase et al., 2009). It was shown that enrichment of histone H3 acetylation at the *Kiss1* promoter is associated with a pubertal increase in *Kiss1* expression in rats (Lomniczi et al, 2013). ARC *Kiss1* expression is downregulated by estrogen-dependent histone deacetylation in adult mice (Tomikawa et al., 2012). Thus, prepubertal estrogen may decrease histone H3 acetylation at the ARC *Kiss1* promoter, resulting prepubertal suppression of *Kiss1* expression.

Interpretation of the current results for mPOA estradiol microimplants is very challenging. This is because a large number of neurons, such as GABAergic (Flugge et al., 1986; Herbison AE, 1997), glutamatergic (Eyigor et al., 2004), dopaminergic (Yuri et al., 1994), and several peptidergic (Herbison et al., 1992; Yuri et al., 1994; Bloch et al., 1992; Herbison et al., 1992; Axelson et al., 1992; Simerly et al., 1996) neurons, have been reported to express ERα in the mPOA. The involvement of these neurons in prepubertal restraint of GnRH/LH secretion remains elusive. It has been suggested that an increase in glutamatergic transmission precedes the pubertal decrease in GABAergic inhibitory input to the GnRH neurons (Ojeda et al., 2003). In support of this, previous studies showed that an increase in hypothalamic GABA content, which is induced by a GABA-transaminase

inhibitor, suppressed gonadotropin secretion and puberty onset in female rats (Feleder et al., 1999) and that a GABAA receptor antagonist stimulated gonadotropin secretion in female rats (Mitsushima et al., 1997). On the other hand, it has also been suggested that an increase in GABAergic tone is involved in a prepubertal increase in GnRH secretion, because a GABAA receptor agonist stimulated gonadotropin secretion in prepubertal female rats (Feleder et al., 1996; Sacchi et al., 1998). One possibility suggested by the current results using mPOA estradiol microimplants is that mPOA estrogen-responsive neurons, which may be GABAergic or other neurons, exert an inhibitory influence on ARC kisspeptin neuronal activity. If this is the case, ARC kisspeptin neuronal activity might be suppressed by estrogen both in direct and indirect manners. Further studies, e.g., projection of mPOA estrogen-responsive neurons into the ARC kisspeptin neurons, are required to clarify this issue.

In conclusion, the results obtained from the present study suggest that estrogen-responsive neurons located in the mPOA and ARC play key roles in estrogen-dependent prepubertal restraint of GnRH/LH secretion in female rats.

Figures

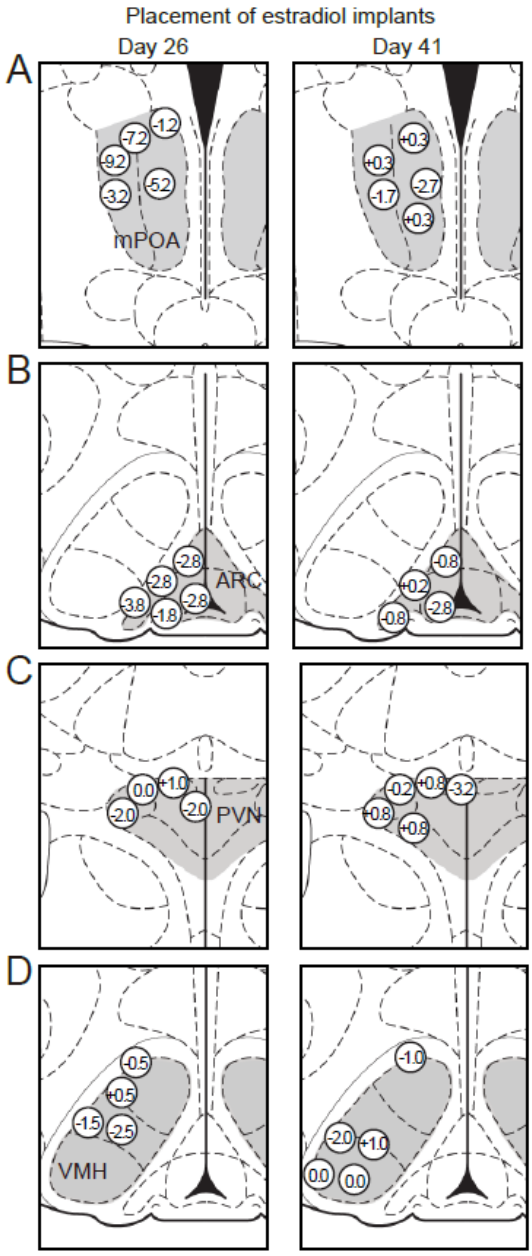

Fig. 5. Schematic drawings of the medial preoptic area (mPOA, A), hypothalamic arcuate nucleus (ARC, B), paraventricular nucleus (PVN, C) and ventromedial nucleus (VMH, D), illustrating the placement of estradiol microimplants in each animal sacrificed at 26 (left panels) and 41 (right panels) days of age. Encircled numbers indicate changes in the number of luteinizing hormone (LH) pulses during the 3-h sampling period induced by estradiol microimplants, compared with the mean LH pulse frequency in cholesterol-implanted controls.

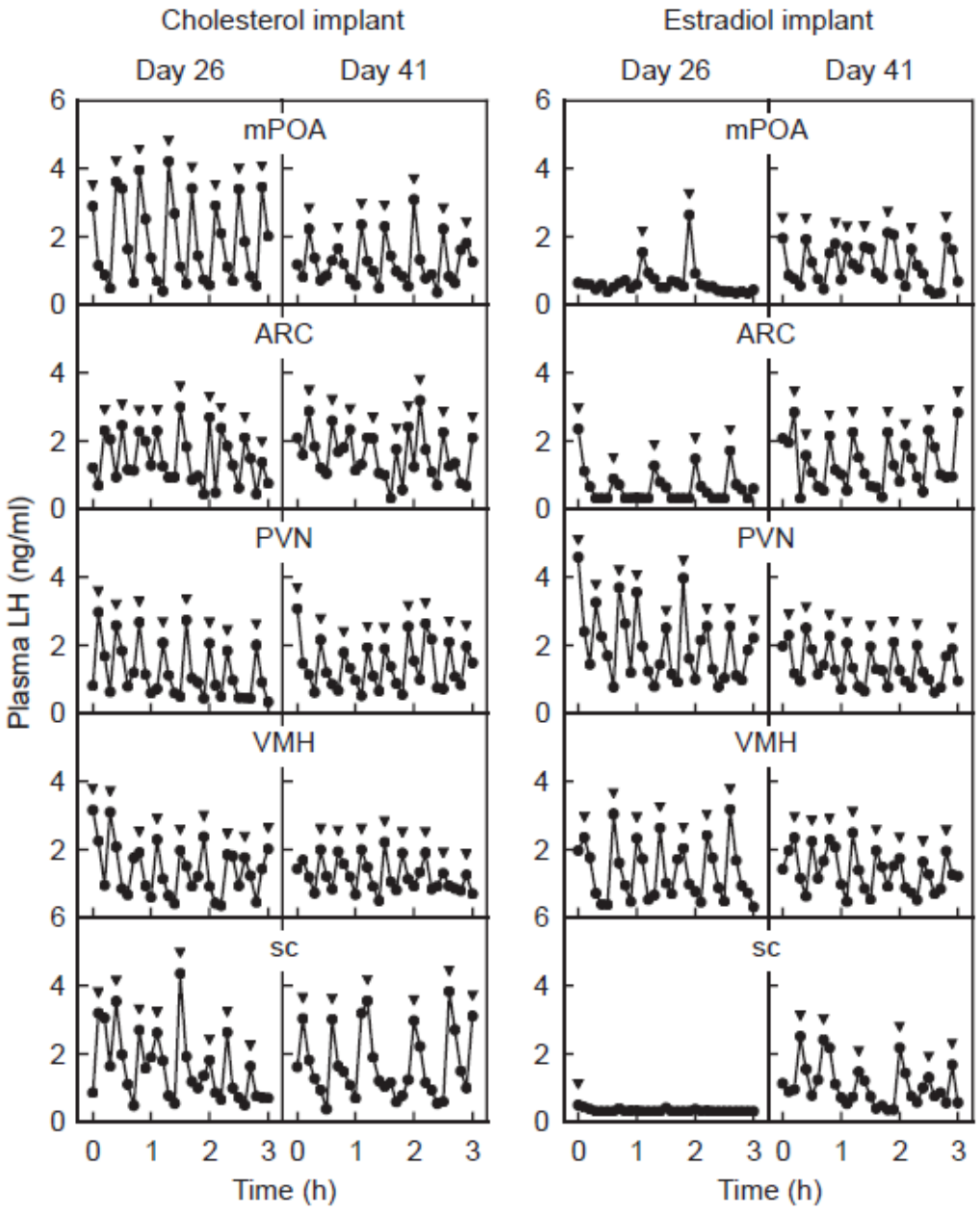

Fig. 6. Plasma LH profiles in representative cholesterol- (left panel) or estradiol-implanted (right panel) ovariectomized (OVX) rats at 26 (prepubertal period) and 41 (postpubertal period) days of age. Estradiol or cholesterol was implanted into the mPOA, ARC, PVN or VMH in OVX rats for 6 days. Some OVX animals received subcutaneous (sc) estradiol treatment for 6 days. Arrowheads indicate LH pulses identified with the PULSAR computer program.

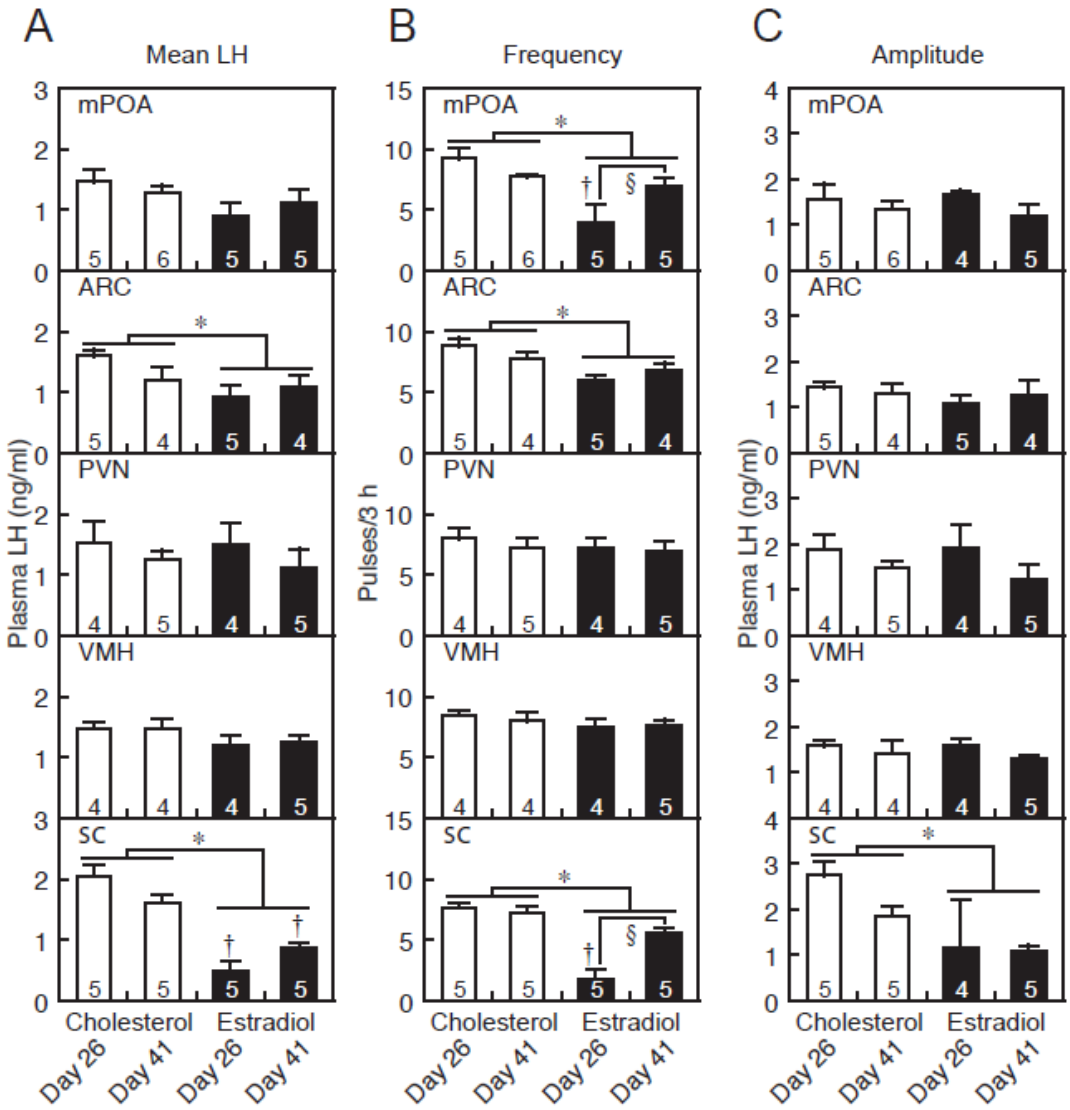

Fig. 7. Mean plasma LH concentrations (A) and the frequency (B) and amplitude (C) of LH pulses in cholesterol- (left panel) or estradiol-implanted (right panel) OVX rats at 26 (prepubertal period) and 41 (postpubertal period) days of age. Estradiol or cholesterol was implanted into the mPOA, ARC, PVN or VMH in OVX rats for 6 days. Some OVX animals received sc estradiol treatment for 6 days. Values are means ± SEM. Numbers in each column indicate the number of animals used. *Significant main effect of estradiol treatment ($P<0.05$, two-way ANOVA). †Significant difference between cholesterol- and estradiol-implanted OVX rats within each period ($P<0.05$, two-way ANOVA). §Significant difference between the pre- and postpubertal periods within estradiol-implanted OVX rats ($P<0.05$, two-way ANOVA).

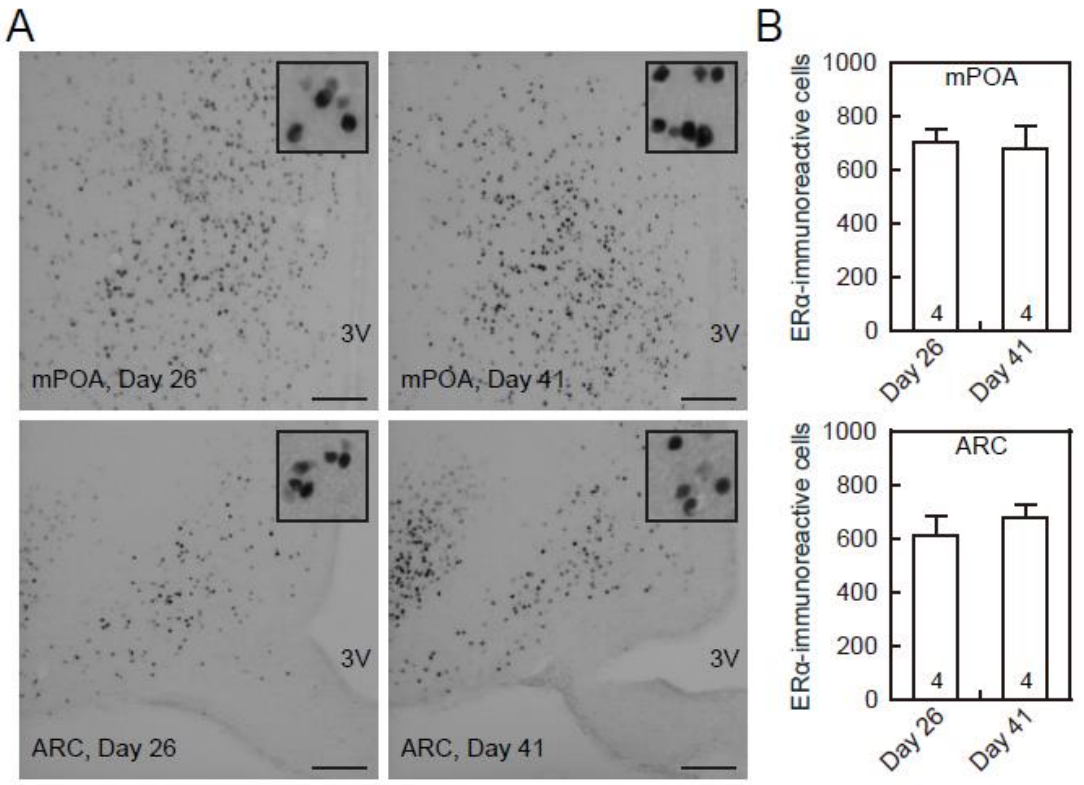

Fig. 8. Estrogen receptor α (ERα)-immunoreactive (ir) cells in OVX rats. A, Photomicrographs of ERα-ir cells in the mPOA and ARC at 26 and 41 days of age. Scale bars, 100 μm. B, Numbers of ERα-ir cells in the mPOA and ARC. Numbers of ERα-ir cells were not significantly different between 26 and 41 days of age. Values are mean ± SEM. Numbers in each column indicate the number of animals used.

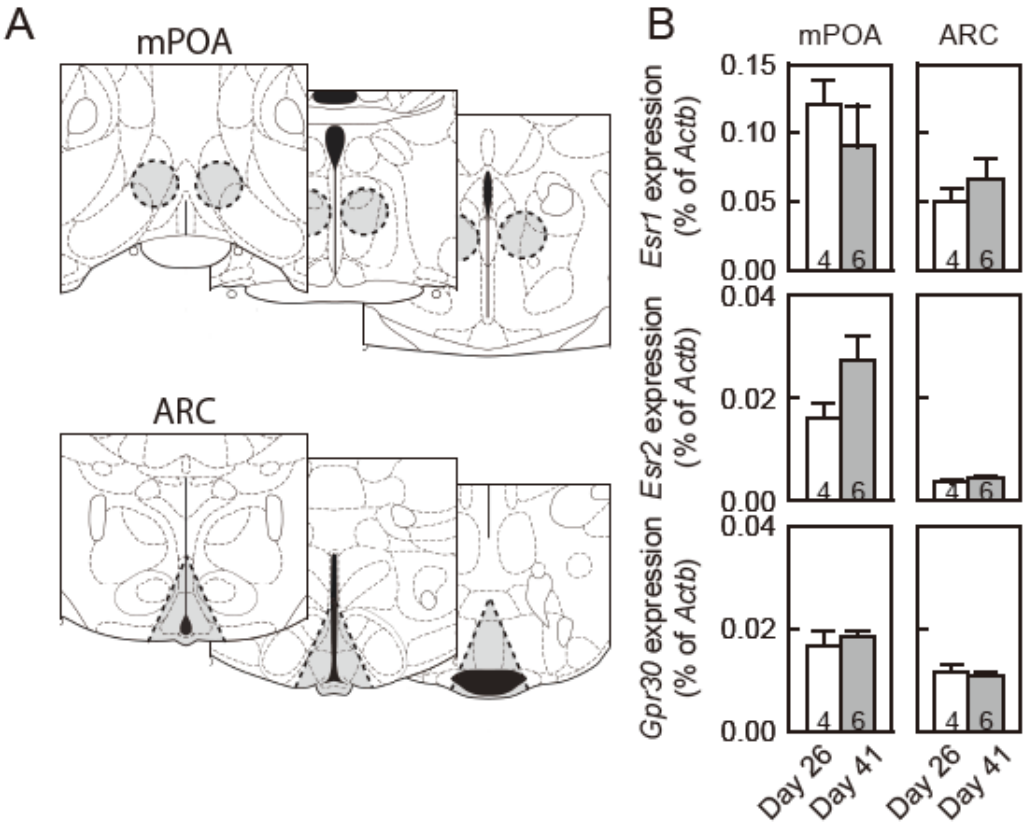

Fig. 9. Messenger RNA expression for three estrogen receptors in the mPOA and ARC in OVX rats. A, The schematic drawings show dissection or punch-out sites for the mPOA (upper panels) and ARC-median eminence (lower panels) regions (surrounded by dotted lines) from the anterior to posterior end. B, *Esr1*, *Esr2* and *Gpr30* mRNA expression. Values are normalized to the ratio to the copy number of *Actb* transcripts and presented as means ± SEM. Numbers in each column indicate the number of animals used.

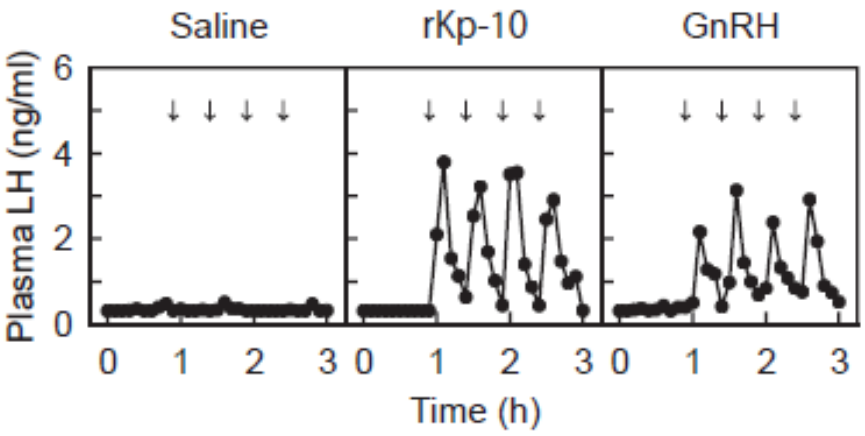

Fig. 10. Effects of the C-terminal decapeptide of rat kisspeptin (rKp-10, 10 nmol/kg body weight) and gonadotropin-releasing hormone (GnRH, 0.1 nmol/kg) on plasma LH levels in representative subcutaneous estradiol-treated OVX rats. Arrows indicate intravenous injection of rKp-10, GnRH or saline.

Chapter 2: Evaluation of pharmacokinetics / pharmacodynamics and efficacy of one-month depots of Kiss-001 and Kiss-002, investigational kisspeptin analogs, in male rats and an androgen-dependent prostate cancer model

Abstract

Kiss-001 and Kiss-002 are kisspeptin agonist analogs with improved *in vivo* stability and activity. Previous studies showed that continuous subcutaneous administration of Kiss-001 or Kiss-002 caused rapid and profound reductions in plasma testosterone levels in various species, including male healthy volunteers, suggesting their therapeutic potential as anti-prostate cancer agents. For clinical drug development, one-month sustained-release depots of Kiss-001 and Kiss-002, Kiss-001-SR(1M) and Kiss-002-SR(1M), were designed to improve usability in clinical practice. In this study, the pharmacokinetics/pharmacodynamics (PK/PD) profiles of Kiss-001-SR(1M) and Kiss-002-SR(1M) were initially tested in male rats to ensure their eligibility as one-month depots. The therapeutic advantages of Kiss-001-SR(1M) and Kiss-002-SR(1M) over TAP-144-SR(1M), a GnRH agonist which is currently used in the treatment of prostate cancer, were then investigated in a JDCaP xenograft rat model. Kiss-001-SR(1M) and Kiss-002-SR(1M) maintained certain levels of plasma Kiss-001 free form (Kiss-001F) and plasma Kiss-002 free form (Kiss-002F) for at least 4 weeks, before clearance from the circulation. Accompanying their desirable PK profiles, Kiss-001-SR(1M) and Kiss-002-SR(1M) showed favorable PD responses as one-month depots and demonstrated better testosterone control than TAP-144-SR(1M). Both depots exerted rapid and profound suppression of plasma testosterone levels in male rats. These profound suppressive effects were maintained in dose-dependent manners, before recovery toward normal levels. In the JDCaP xenograft model, Kiss-001-SR(1M) and Kiss-002-SR(1M) both showed better prostate-specific antigen (PSA) control than TAP-144-SR(1M), although all treatment groups eventually experienced PSA recurrence and tumor regrowth.

In conclusion, this study demonstrates that both Kiss-001-SR(1M) and Kiss-002-SR(1M) have desirable and better PK/PD profiles than TAP-144-SR(1M) in rats, which could potentially provide better clinical outcomes in androgen-dependent prostate cancer.

Introduction

Prostate cancer is a globally common cancer in men. Although the cancer death rates in the United States have continued to decline recently, prostate cancer still exhibits high morbidity and mortality in elderly men (Edwards et al., 2014). As prostate cancer primarily grows in an androgen-dependent manner, androgen deprivation therapy (ADT) has been the gold standard for treatment of advanced prostate cancer (Mohler et al., 2019). Various ADT regimens have been developed to date, including surgical castration and medical castration with gonadotropin-releasing hormone (GnRH) agonist or antagonist alone or in combination with androgen receptor antagonist.

Kisspeptin (also known as metastin) is the endogenous ligand for the human G-protein-coupled receptor KISS1R (also known as GPR54) (Ohtaki et al., 2001). Kisspeptin plays a key role in reproductive function through stimulation of GnRH release. In general, male reproductive function is centrally regulated through the hypothalamus–pituitary–gonadal axis. GnRH released from the hypothalamus stimulates the pituitary gland to secrete two gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH or FSH plays an important role in testicular function, particularly in testosterone synthesis and spermatogenesis. Indeed, acute administration of kisspeptin was shown to activate male reproductive function in a broad range of mammalian species including humans (Gottsch et al., 2004; Thompson et al., 2004; Dhillo et al., 2005; Shahab et al., 2005; Ohkura et al., 2009; George et al., 2011). In contrast, chronic administration of kisspeptin was reported to suppress reproductive function in adult male rats (Thompson et al., 2006) and agonadal juvenile male monkeys (Seminara et al., 2006; Ramaswamy et al., 2007).

Kiss-001 (another name TAK-448) and Kiss-002 (another name TAK-683) are investigational kisspeptin analogs that have improved metabolic stability with maintained potent agonistic activity for KISS1R compared with the natural peptide (Asami et al., 2013). It was previously reported that continuous subcutaneous administration of Kiss-001 or Kiss-002 induced transient increases in plasma testosterone, followed by more rapid and profound plasma testosterone reductions compared with TAP-144 (leuprolide, a GnRH agonist) in male rats (Matsui et al., 2014). Similar profound testosterone-lowering effects were observed in dogs, monkeys, and human male healthy volunteers (Tanaka et al., 2010; Scott et al., 2013; MacLean et al., 2014). Furthermore, chronic Kiss-001 and Kiss-002 administration showed good therapeutic responses with prostate-specific antigen (PSA) reductions in the human androgen-dependent prostate cancer (ADPC) JDCaP xenograft rat model (Matsui et al., 2014) and prostate cancer patients (MacLean et al., 2014). These findings suggest that chronic administration of Kiss-001 or Kiss-002 may hold promise as novel therapeutic agents for the treatment of prostate cancer.

Kiss-001-SR(1M) and Kiss-002-SR(1M) were recently developed as one-month sustained-release depots of Kiss-001 and Kiss-002, by applying the drug delivery technology for TAP-144-SR(1M) (Toguchi et al., 1991). In this study, I determined the pharmacodynamics (PD) and pharmacokinetics (PK) of Kiss-001-SR(1M) and Kiss-002-SR(1M) in intact male Sprague-Dawley rats, and then investigated their therapeutic advantages over TAP-144-SR(1M) in the JDCaP xenograft rat model.

Materials and Methods

Materials

Once-a-month injectable sustained-release depots of Kiss-001, Kiss-002, and leuprolide acetate, designated Kiss-001-SR(1M), Kiss-002-SR(1M), and TAP-144-SR(1M), and their vehicle solution (Lupron vehicle 2ML) were manufactured at Takeda Pharmaceutical Company Ltd. (Osaka, Japan). Kiss-001 and Kiss-002 used for the depots were also manufactured by Takeda pharmaceutical company Ltd with good laboratory practice standard. Aprotinin (Bayer Health Care Pharmaceuticals, Leverkusen, Germany) and EDTA-2Na (Dojindo Laboratories, Kumamoto, Japan) were used for plasma sampling.

Animals

Adult male Sprague-Dawley Crl:CD(SD) rats (Sprague-Dawley rats; 8 weeks of age) were purchased from Charles River Laboratories Japan (Kanagawa, Japan). Male F344/N Jcl-rnu/rnu rats (nude rats; 5 weeks of age) were purchased from CLEA Japan (Tokyo, Japan). All animals were housed in a room with controlled temperature and humidity on a 12-h/12-h light/dark cycle, and had free access to food and water. All animal experiments were approved by Experimental Animal Care and Use Committee.

PK/PD profiles of Kiss-001-SR(1M) and Kiss-002-SR(1M) in male Sprague-Dawley rats

Two separate experiments were conducted to determine the PK/PD profiles of Kiss-001-SR(1M) or Kiss-002-SR(1M) in male Sprague-Dawley rats, and then each PD profile

was compared with that of TAP-144-SR(1M). In both experiments, male Sprague-Dawley rats were evenly divided into 10 groups (n = 14 per group) based on body weight after habituation for 1 week or more. Kiss-001-SR(1M), Kiss-002-SR(1M), or TAP-144-SR(1M) was suspended in the Lupron vehicle 2ML on the day of dosing (day 0). The doses tested in this study were determined based on the results in preliminary studies for formulation optimization. The doses of each suspended formulation were calculated as the free form of each active pharmaceutical ingredient (Kiss-001F, Kiss-002F, and leuprolide): Experiment 1, Kiss-001-SR(1M) 0.03, 0.1, 0.3, and 1.0 mg/kg versus TAP-144-SR(1M) 0.3, 1, 3, and 10 mg/kg; Experiment 2, Kiss-002-SR(1M) 0.29, 0.96, 2.9, and 9.6 mg/kg versus TAP-144-SR(1M) 0.29, 0.95, 2.9, and 9.5 mg/kg. In one of the groups in each experiment, all rats underwent a bilateral orchiectomy (ORX) performed on day 0 as a positive control. Blood samples were obtained via the tail vein, mixed with aprotinin solution containing 10% (wt/vol) EDTA-2Na, and centrifuged to obtain plasma samples for measurement of plasma testosterone and test compound levels. Half of the animals in each group were euthanized on day 28 to measure the weights of the prostate, seminal vesicles, and testes to assess the impact of Kiss-001-SR(1M), Kiss-002-SR(1M), or TAP-144-SR(1M) on reproductive organs. The weights of the prostate, seminal vesicles, and testes were expressed as relative weights compared to each animal's body weight (percent body weight).

Efficacy study in the JDCaP subrenal capsule xenograft rat model

The JDCaP xenograft model was maintained in nude mice as previously described (Kimura et al., 2009). At the beginning of the study, a small piece of JDCaP xenograft was transplanted under the subrenal capsule of each male nude rat, and its growth was

monitored by measuring serum PSA levels. Rats who showed a sufficient PSA increase were selected and assigned to the following 10 groups based on their PSA levels: control, ORX, Kiss-001-SR(1M) 0.1, 0.3, and 1 mg/kg, Kiss-002-SR(1M) 1, 3, and 10 mg/kg, and TAP-144-SR(1M) 3 and 10 mg/kg. After initiation of each treatment, serum PSA levels were sequentially measured to monitor the tumor growth. The animals were euthanized when their serum PSA levels exceeded a threshold of 20 ng/ml or on day 280.

Measurements

Plasma PSA levels were determined using a MARKIT-M PA ELISA Kit (Dainippon Sumitomo Pharma, Osaka, Japan), according to the manufacturer's protocol. The detection range for PSA was 0.5-100 ng/ml. The coefficient variance was below 10%. For determination of plasma testosterone levels, a DPC-Total Testosterone RIA Kit (Mitsubishi Chemical Medience Corporation, Tokyo, Japan) was used. The lower limit of quantification (LLOQ) for PSA and testosterone was 0.5 and 0.04 ng/ml, respectively. Values below the LLOQ were presumed to be each LLOQ value. The concentrations of plasma free forms of Kiss-001 (Kiss-001F) and Kiss-002 (Kiss-002F) were determined by ELISA at Takeda Analytical Research Laboratories Ltd. (Osaka, Japan). The LLOQ for Kiss-001F and Kiss-002F was 3 and 5 pg/ml, respectively. Values below the LLOQ were presumed to be 0.

Statistics

Statistical significance was analyzed using SAS software version 8.2 (SAS Institute Inc., Cary, NC). Data were evaluated as mean ± standard deviation (S.D.) unless otherwise stated. Homogeneity of variance for multiple groups was analyzed by the F test

(two groups) or Bartlett's test (multiple groups), and differences were analyzed by the Wilcoxon rank sum test, one-tailed Shirley–Williams test, or one-tailed Williams' test. Differences were considered significant when the P-values were ≤0.01 (Wilcoxon rank sum test) or ≤0.025 (one-tailed Williams' or Shirley–Williams test).

Results

PK/PD profile of Kiss-001-SR(1M) and Kiss-002-SR(1M) in male Sprague-Dawley rats

To ensure that Kiss-001-SR(1M) and Kiss-002-SR(1M) have suitable pharmacological profiles as one-month sustained-release formulations, the PK/PD profile of each depot was determined in male Sprague-Dawley rats. Kiss-001-SR(1M) exhibited a stable sustained-release PK profile for 28 days at each dose tested (Fig. 11). The plasma Kiss-001F level on day 7 in the Kiss-001-SR(1M) 0.03, 0.1, 0.3, and 1.0 mg/kg dosing groups was 14.5 ± 12.6, 31.4 ± 25.4, 164.0 ± 285.5, and 248.6 ± 152.4 pg/ml, respectively, and these levels were maintained until day 28. After that, the plasma Kiss-001F levels declined and reached <30 pg/ml on days 35 in the Kiss-001-SR(1M) 0.1 and 0.3 mg/kg dosing groups and days 42 in 1.0 mg/kg dosing group, respectively. Kiss-002-SR(1M) also exhibited a sustained-release profile in male rats. The plasma Kiss-002F levels in the Kiss-002-SR(1M) at 0.29, 0.96, 2.9, and 9.6 mg/kg dosing groups gradually declined from day 7 (7.4 ± 12.8, 135.3 ± 53.1, 584.4 ± 253.8, and 2074.9 ± 784.6 pg/ml, respectively) to day 21 (2.2 ± 6.8, 11.4 ± 17.5, 93.2 ± 73.9, and 322.0 ± 190.5 pg/ml, respectively), and then remained stable in each dosing group until day 35. The plasma Kiss-002F levels were maintained at >50 pg/ml until days 7, 35, and 42 in the Kiss-002-SR(1M) 0.96, 2.9, and 9.6 mg/kg dosing groups, respectively.

Fig. 12 and 13 show the time-dependent changes in plasma testosterone levels in each treatment group. The vehicle group showed stable plasma testosterone levels throughout the study period. In contrast, the ORX group showed complete suppression of plasma testosterone levels (below the LLOQ) on the next day after dosing, and the

suppressed levels were maintained throughout the study period. All dosing groups for Kiss-001-SR(1M) and Kiss-002-SR(1M) showed transient testosterone increases, followed by abrupt plasma testosterone reductions by day 3. The durations of the suppressive effects of Kiss-001-SR(1M) and Kiss-002-SR(1M) were maintained in dose-dependent manners. Kiss-001-SR(1M) 0.1 mg/kg and above and Kiss-002-SR(1M) 9.6 mg/kg achieved continuous complete testosterone suppression until day 28, before recovery to normal levels. TAP-144-SR(1M), as the comparator, also showed dose-dependent plasma testosterone suppression following a transient increase on day 1. Although TAP-144-SR(1M) at the highest doses (9.5 and 10 mg/kg) showed maximum plasma testosterone suppression in both experiments, the nadir values of the plasma testosterone levels were still detectable (day 21: 0.060 ± 0.075 and 0.072 ± 0.073 ng/ml, respectively).

The plasma testosterone levels and organ weights of the testes, ventral prostate glands, and seminal vesicles on day 28 are shown in Fig. 14 and 15. Kiss-001-SR(1M) and Kiss-002-SR(1M) showed significant reductions in plasma testosterone levels and reproductive organ weights in dose-dependent manners on day 28. The magnitudes of the reductions in prostate gland and seminal vesicle weights for Kiss-001-SR(1M) 0.1 mg/kg and above, and Kiss-002-SR(1M) 9.6 mg/kg were comparable to those for ORX. Meanwhile, TAP-144-SR(1M) caused significant decreases in plasma testosterone levels and organ weights, but its effects were weaker than those of ORX. These lowering effects on plasma testosterone levels and organ weights were recovered toward the normal levels until day 84 (Fig. 16 and 17).

Comparison of antitumor activities of Kiss-001-SR(1M), Kiss-002-SR(1M), and TAP-144-SR(1M) in the JDCaP subrenal capsule xenograft rat model

To characterize the therapeutic potential of Kiss-001-SR(1M) and Kiss-002-SR(1M) for the treatment of prostate cancer, their antitumor effects were investigated and compared with that of TAP-144-SR(1M) in the JDCaP subrenal capsule xenograft rat model. Initially, all groups had 6 animals, but the numbers were decreased by deaths or euthanasia for PSA recurrence (Fig. 18).

The serum PSA levels in the vehicle group gradually increased, while those in the ORX group immediately decreased below the LLOQ (0.5 ng/ml) after treatment initiation (Fig. 18 and 19). All tested doses of Kiss-002-SR(1M), except for Kiss-002-SR(1M) 1 mg/kg, and Kiss-001-SR(1M) rapidly decreased serum PSA levels within 8 days after first dosing and then maintained the PSA levels below the LLOQ. TAP-144-SR(1M) 3 and 10 mg/kg also decreased serum PSA levels following a transient increase, but required approximately 4 weeks to achieve complete PSA suppression.

All treatment groups except for Kiss-002-SR(1M) 1 mg/kg showed continuously suppressed serum PSA levels after treatment initiation, although biochemical recurrence was eventually observed. The serum PSA levels in the TAP-144-SR(1M) 3 and 10 mg/kg groups began to increase at around 5 months after treatment initiation. In contrast, it required approximately 6 months for biochemical recurrence in the ORX, Kiss-001-SR(1M) 1, 3, and 10 mg/kg, and Kiss-002-SR(1M) 3 and 10 mg/kg treatment groups.

Discussion

In previous studies, continuous subcutaneous administration of Kiss-001 or Kiss-002 was reported to induce more rapid and profound plasma testosterone reductions than TAP-144 in various species including human male healthy volunteers (Tanaka et al., 2010; Scott et al., 2013; MacLean et al., 2014). Although these findings strongly suggested that Kiss-001 or Kiss-002 could become novel therapeutic agents for ADPC, the dosing method was a limitation to their drug development given the clinical setting. To improve the usability in clinical practice, I planned to design one-month sustained-release depots of Kiss-001 and Kiss-002 by applying the original drug delivery technology for TAP-144-SR(1M) (Toguchi et al., 1991). In the present study, I succeeded in developing one-month depots with desirable PK/PD profiles, designated Kiss-001-SR(1M) and Kiss-002-SR(1M), and then demonstrated their therapeutic advantages over TAP-144-SR(1M) in the JDCaP xenograft rat model.

Kiss-001-SR(1M) and Kiss-002-SR(1M) exhibited favorable PK profiles in male rats. Both depots maintained certain levels of plasma Kiss-001F or Kiss-002F for 4–5 weeks, before clearance from the circulation. Accompanying these PK profiles, Kiss-001-SR(1M) and Kiss-002-SR(1M) showed desirable PD responses as one-month depots in male rats. Kiss-001-SR(1M) and Kiss-002-SR(1M) showed rapid and profound suppression of plasma testosterone levels within 3 days after dosing at all doses tested. This profound plasma testosterone suppression was maintained in dose-dependent manners. Kiss-001-SR(1M) at 0.1 mg/kg and above, and Kiss-002-SR(1M) at 9.6 mg/kg achieved continuous complete testosterone suppression until day 28, followed by recovery to normal levels. In contrast, TAP-144-SR(1M), as the comparator, showed

continuous suppression of plasma testosterone levels in a dose-dependent manner, but the initial transient increases in plasma testosterone level were longer than those with Kiss-001-SR(1M) and Kiss-002-SR(1M), and the nadir values of the plasma testosterone level remained above the LLOQ even in the maximum dosing group (10 mg/kg). The differences in the plasma testosterone profiles were obviously reflected in the sexual organ weights on day 28. Kiss-001-SR(1M) and Kiss-002-SR(1M) showed significant reductions in the weights of the testes, ventral prostate gland, and seminal vesicles close to the castration level, while TAP-144-SR(1M) at all doses tested showed only marginal reductions in the weights of these organs. The suppressive effects on plasma testosterone levels and organ weights were recovered toward the normal levels at day 84. However, there were still slight but significant differences, particularly in the testes, between Kiss-001-SR(1M) or Kiss-002-SR(1M) and TAP-144-SR(1M). Given that these findings were commonly observed in the kisspeptin analogue and GnRH agonist treatment groups and similar findings were also reported in studies testing human chorionic gonadotropin (Chatani, 2006) and kisspeptin-54 (Thompson et al., 2006) in rats, it can be considered that unrecovered morphological changes and testicular atrophy were induced by discharge of gonadotropin and were not attributed to any unknown kisspeptin-specific effect. All of the above data indicate that both Kiss-001-SR(1M) and Kiss-002-SR(1M) have favorable pharmacological profiles as one-month depots and show better testosterone control than TAP-144-SR(1M).

The durations of the continuous complete suppressive effects of Kiss-001-SR(1M) and Kiss-002-SR(1M) on plasma testosterone levels were determined by the plasma Kiss-001F and Kiss-002F levels. Kiss-001-SR(1M) at 0.1, 0.3, and 1 mg/kg suppressed plasma testosterone levels below the LLOQ until days 28, 35, and 42, respectively. The plasma

Kiss-001F level at each time point was 31.2 ± 15.3, 24.7 ± 18.7, and 23.3 ± 24.2 pg/ml, respectively. These data suggest that the threshold of the plasma Kiss-001F level for continuous plasma testosterone suppression is approximately 20–30 pg/ml. Similarly, Kiss-002-SR(1M) at 0.96, 2.9, and 9.6 mg/kg achieved complete plasma testosterone suppression until days 7, 21, and 49, respectively. The plasma Kiss-002F level at day 7 in the 0.96 mg/kg group and day 21 in the 2.9 mg/kg group was 135.3 ± 53.1 and 93.2 ± 73.9 pg/ml, respectively. Kiss-002-SR(1M) at 2.9 mg/kg showed a transient slight increase in the plasma testosterone level on day 28, and the plasma Kiss-002F level was 52.8 ± 44.9 pg/ml. The plasma testosterone level then decreased to below the LLOQ on day 35, and the plasma Kiss-002F level on that day was 58.5 ± 59.5 pg/ml. Therefore, the threshold of the plasma Kiss-002F level for continuous plasma testosterone suppression is approximately 50 pg/ml. These thresholds are consistent with the previous findings in a subcutaneous continuous administration study that the minimum effective concentration of plasma Kiss-001F and Kiss-002F in male rats was 36.7 ± 12.9 and 65.8 ± 18.8 pg/ml, respectively (Matsui et al., 2014).

To investigate whether better testosterone control with Kiss-001-SR(1M) or Kiss-002-SR(1M) can provide better therapeutic outcomes in ADPC, I tested the therapeutic effects of Kiss-001-SR(1M) and Kiss-002-SR(1M) in comparison with TAP-144-SR(1M) in the JDCaP xenograft rat model. Both Kiss-001-SR(1M) and Kiss-002-SR(1M) rapidly achieved complete suppression of serum PSA levels within 8 days after treatment initiation, while TAP-144-SR(1M) required 4 weeks to achieve the same condition (Fig. 18 and 19). Furthermore, these data suggest that Kiss-001-SR(1M) and Kiss-002-SR(1M) exert better PSA control during the initial phase of treatment than TAP-144-SR(1M). Though some animals were found dead during the study period, the causes were most

plausibly not related to the dosing since I could also find the death even in the ORX group which received no drug treatment. In addition, Kiss-001 and Kiss-002 have been already evaluated in human with excellent safety profile (Scott et al., 2013; MacLean et al., 2014).

All treatment groups eventually showed PSA recurrence in this study. Unexpectedly, Kiss-001-SR(1M) and Kiss-002-SR(1M) demonstrated tendencies to delay the timing of PSA recurrence compared with TAP-144-SR(1M) in this model. Although the study size was too small to reach a concrete conclusion on the timing of PSA recurrence, these preliminary observations supports the hypothesis that better testosterone control with Kiss-001-SR(1M) and Kiss-002-SR(1M) could prolong the biochemical recurrence-free period in ADPC. Further studies are warranted to elucidate clinical significance of Kiss-001-SR(1M) and Kiss-002-SR(1M).

In conclusion, Kiss-001-SR(1M) and Kiss-002-SR(1M) demonstrated desirable PK/PD profiles and better testosterone control than TAP-144-SR(1M) in male rats. Furthermore, both depots showed tendencies toward prolongation of the biochemical recurrence-free period in the JDCaP xenograft rat model, suggesting that Kiss-001-SR(1M) and Kiss-002-SR(1M) might provide better clinical outcomes for the treatment of ADPC.

Figures

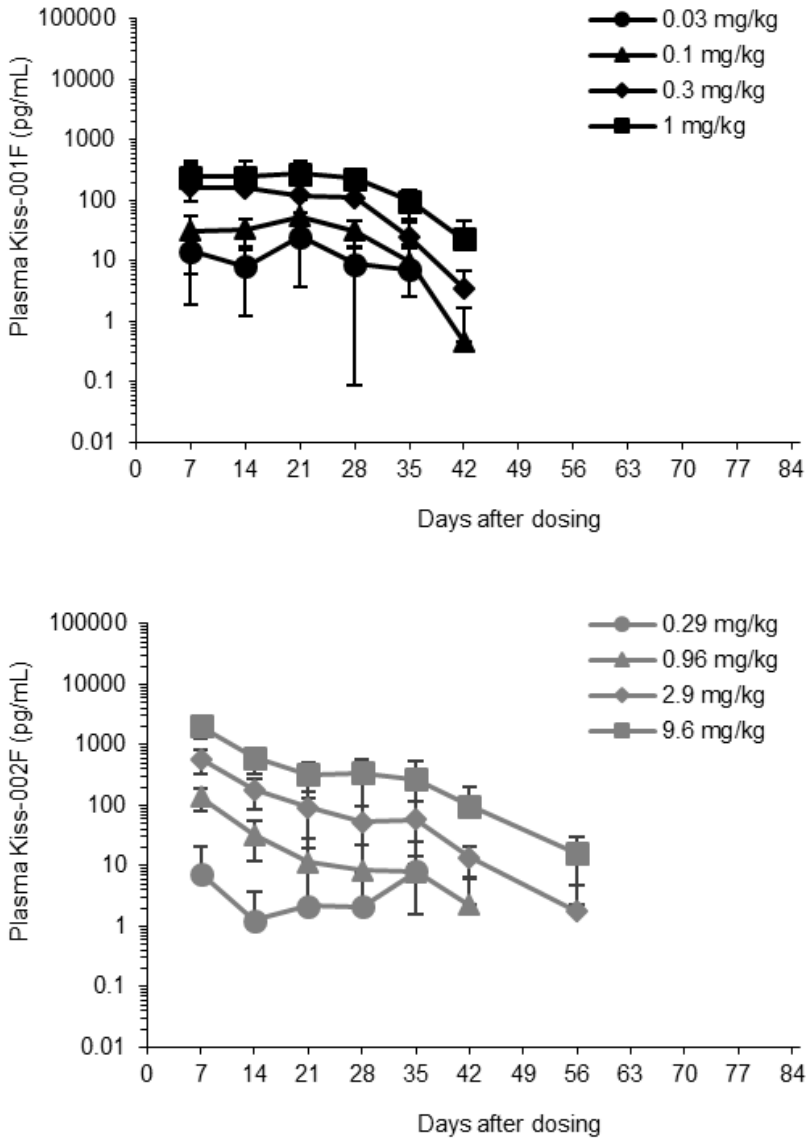

Fig. 11. Plasma Kiss-001F or Kiss-002F levels after single dosing of Kiss-001-SR(1M) or Kiss-002-SR (1M) in male rats. (A) Concentrations of Kiss-001F after Kiss-001-SR(1M) administration. (B) Concentrations of Kiss-002F after Kiss-002-SR(1M) administration. Data represent means ± S.D. (n = 14, days 7–28; n = 7, days 35–84).

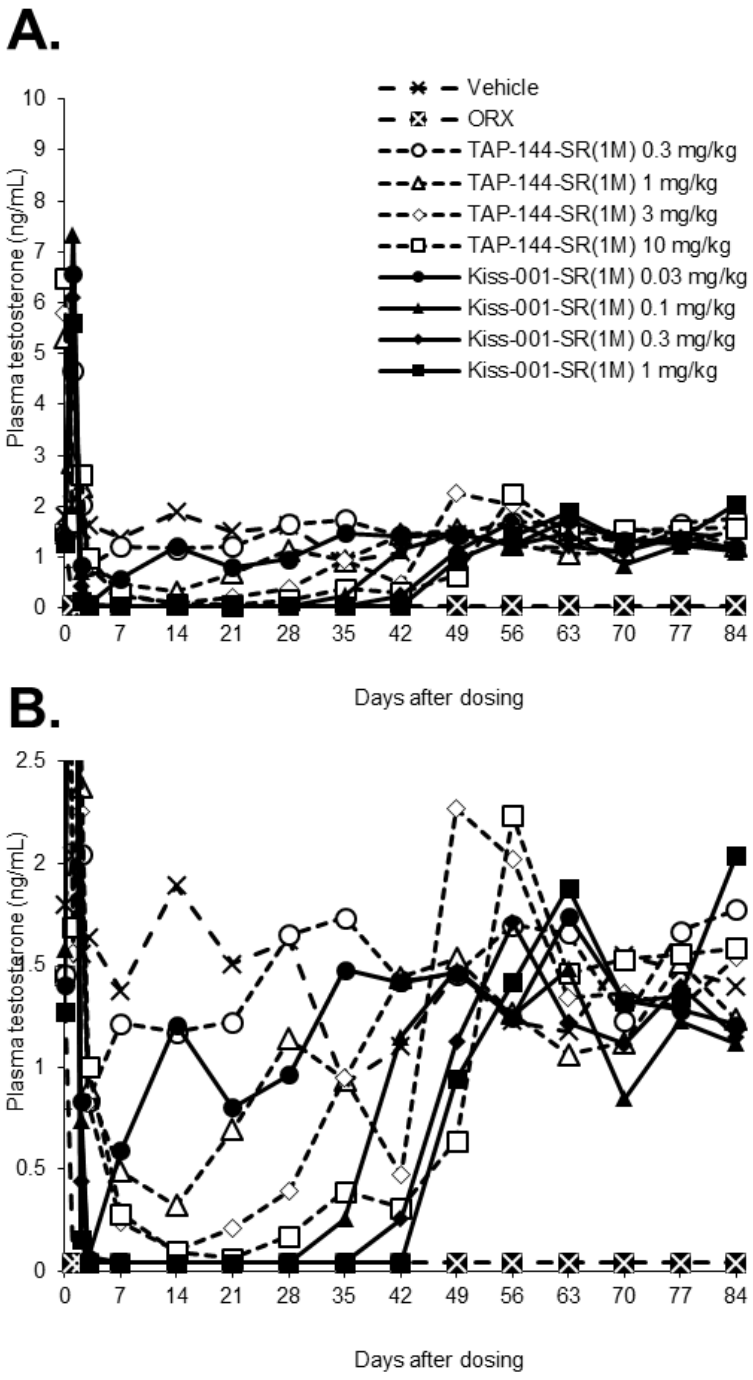

Fig. 12. Changes in plasma testosterone levels after single subcutaneous dosing of Kiss-001-SR(1M) or TAP-144-SR(1M) in adult male rats. (A) Temporal changes in mean plasma testosterone levels (ng/ml). Data represent means ± S.D. (n = 14, days 0–28; n = 7, days 35–84). (B) Same data as in (A), with the vertical maximum scale set at 2.5 ng/ml.

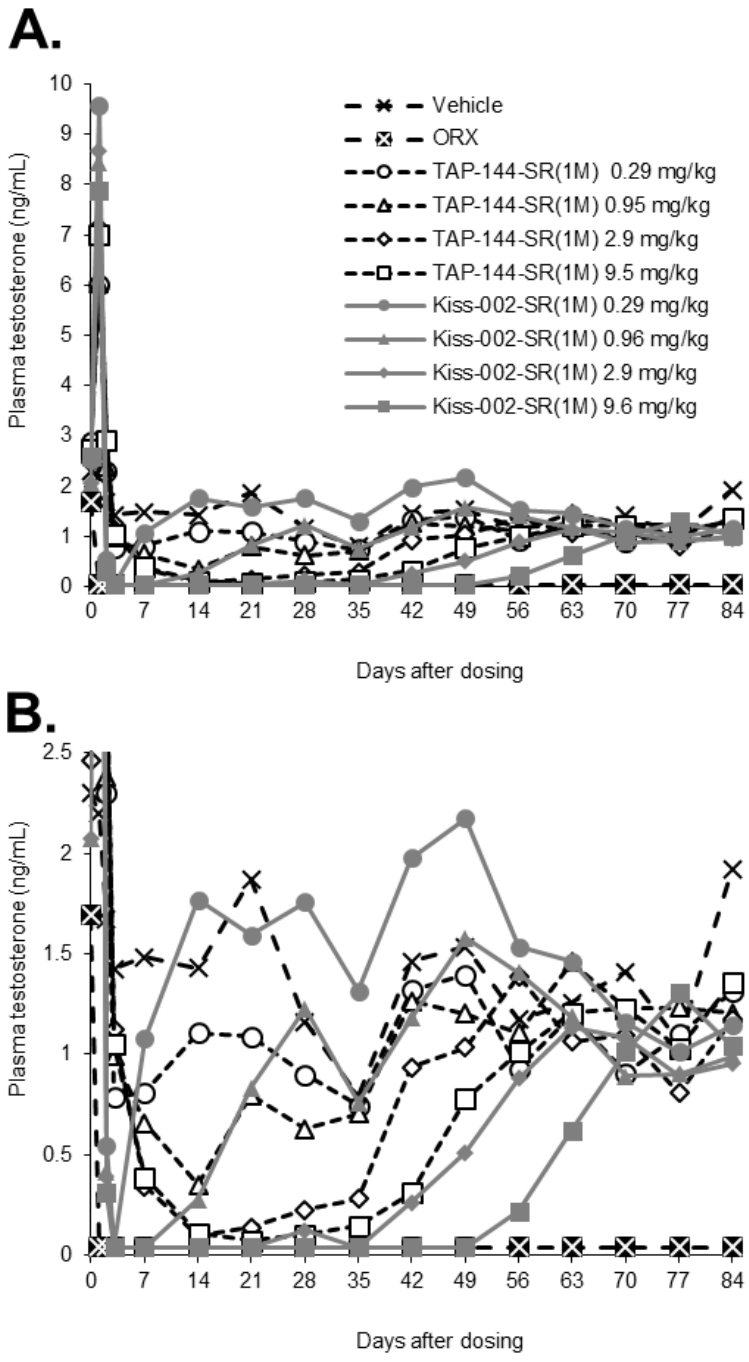

Fig. 13. Changes in plasma testosterone levels after single subcutaneous dosing of Kiss-002-SR(1M) or TAP-144-SR(1M) in adult male rats. (A) Temporal changes in mean plasma testosterone levels (ng/ml). Data represent means ± S.D. (n = 14, days 0–28; n = 7, days 35–84; except TAP-144-SR(1M) 2.9 mg/kg group: n = 14, days 0–3; n = 13, days 7–28; n = 7, days 35–84). (B) Same data as in (A), with the vertical maximum scale set at 2.5 ng/ml.

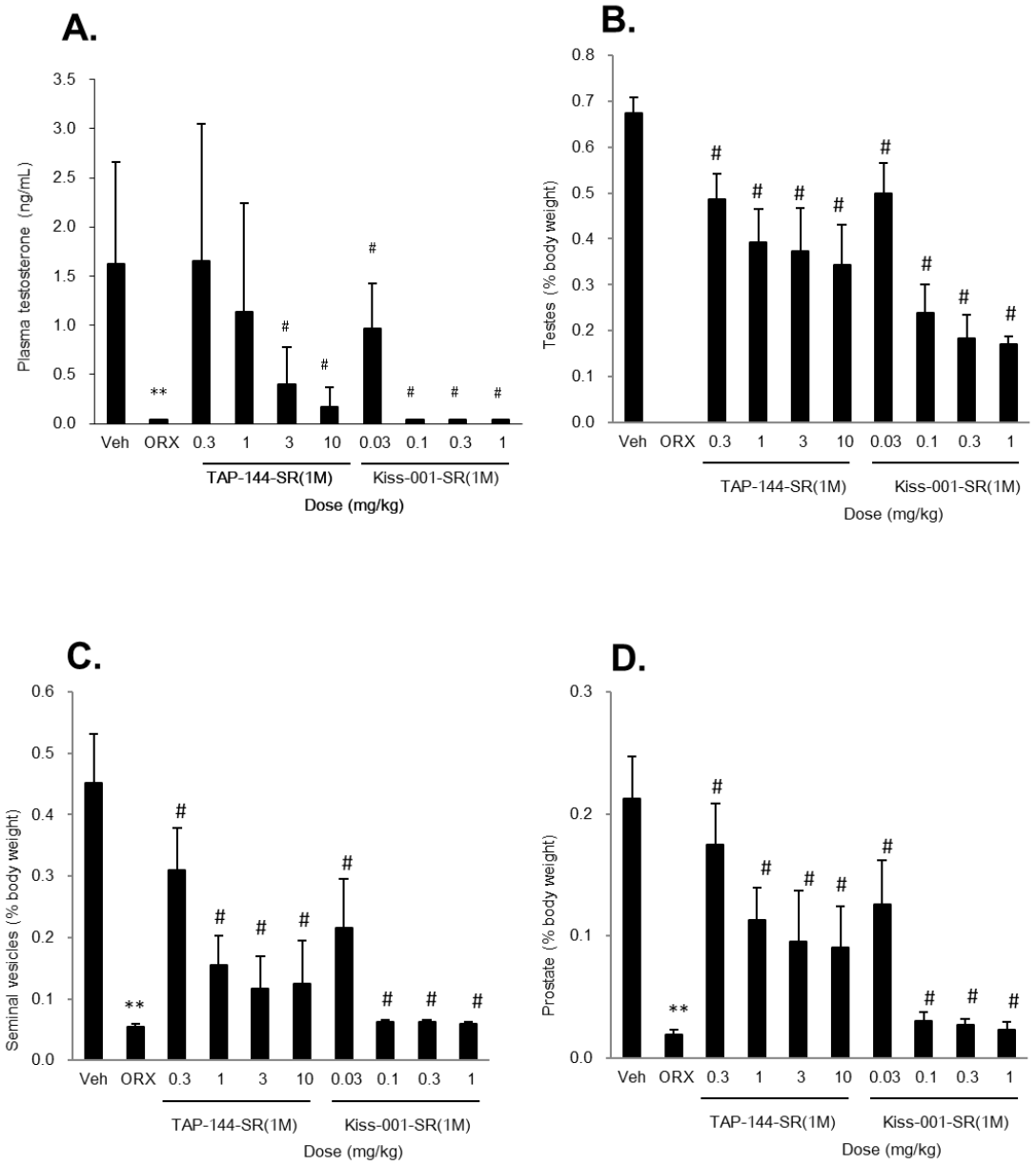

Fig. 14. Plasma testosterone levels and genital organ weights on day 28 after dosing of Kiss-001-SR(1M) or TAP-144-SR(1M) in adult male rats. (A) Plasma testosterone levels on day 28. Data represent means ± S.D. (n = 14). (B–D) Weights of androgen-sensitive testes (B), seminal vesicles (C), and prostates (D) on day 28. Data represent means ± S.D. (n = 7). Veh: vehicle control. $^{**}P \leq 0.01$ vs. vehicle by the Wilcoxon rank sum test; $^{\#}P \leq 0.025$ vs. vehicle by the one-tailed Shirley–Williams test.

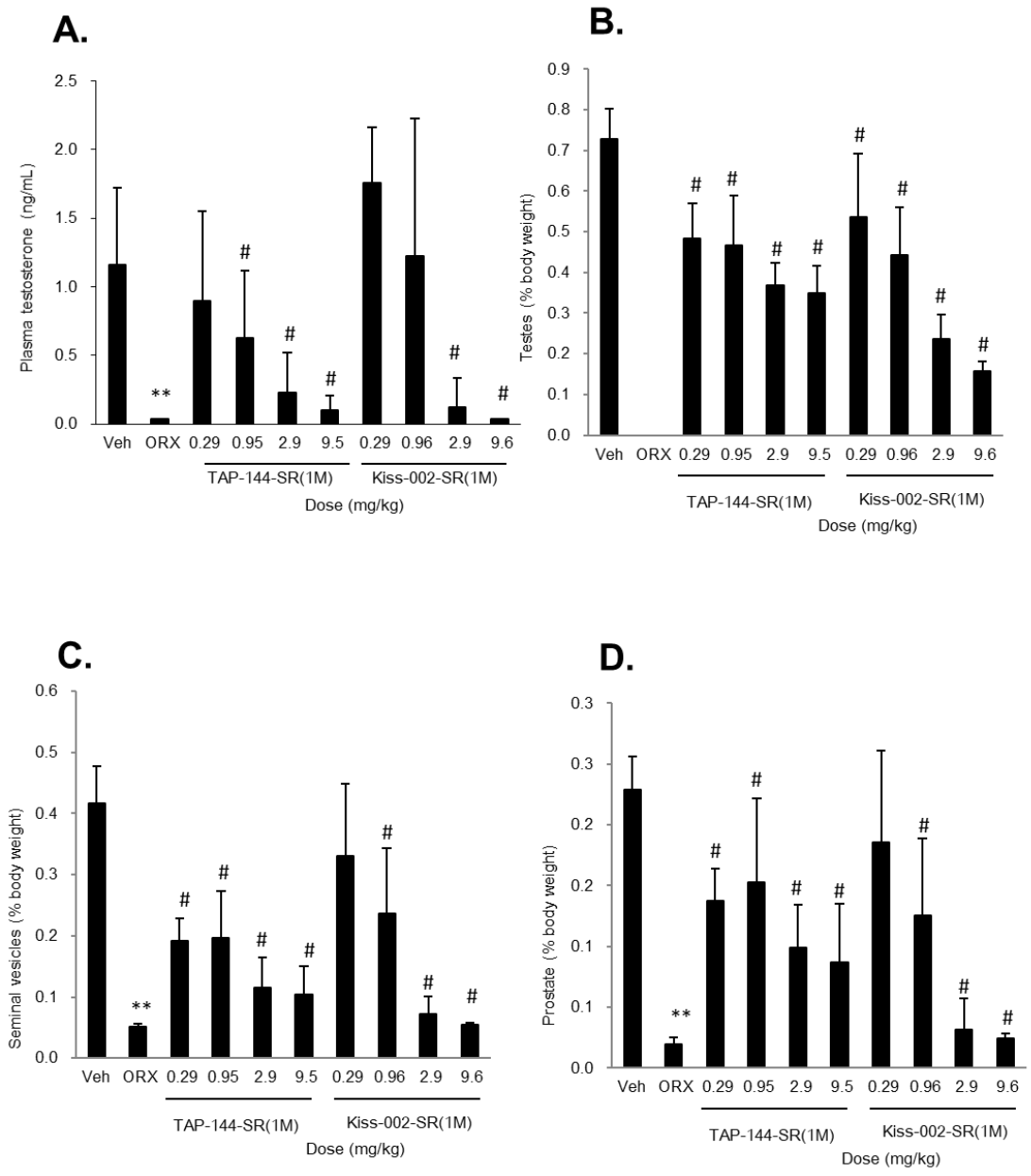

Fig. 15. Plasma testosterone levels and genital organ weights on day 28 after dosing of Kiss-002-SR(1M) or TAP-144-SR(1M) in adult male rats. (A) Plasma testosterone levels on day 28. Data represent means ± S.D. (n = 14). (B–D) Weights of androgen-sensitive testes (B), seminal vesicles (C), and prostates (D) on day 28. Data represent means + S.D. (n = 7). Veh: vehicle control. $^{**}P \leq 0.01$ vs. vehicle by the Wilcoxon rank sum test; $^{\#}P \leq 0.025$ vs. vehicle by the one-tailed Shirley–Williams test.

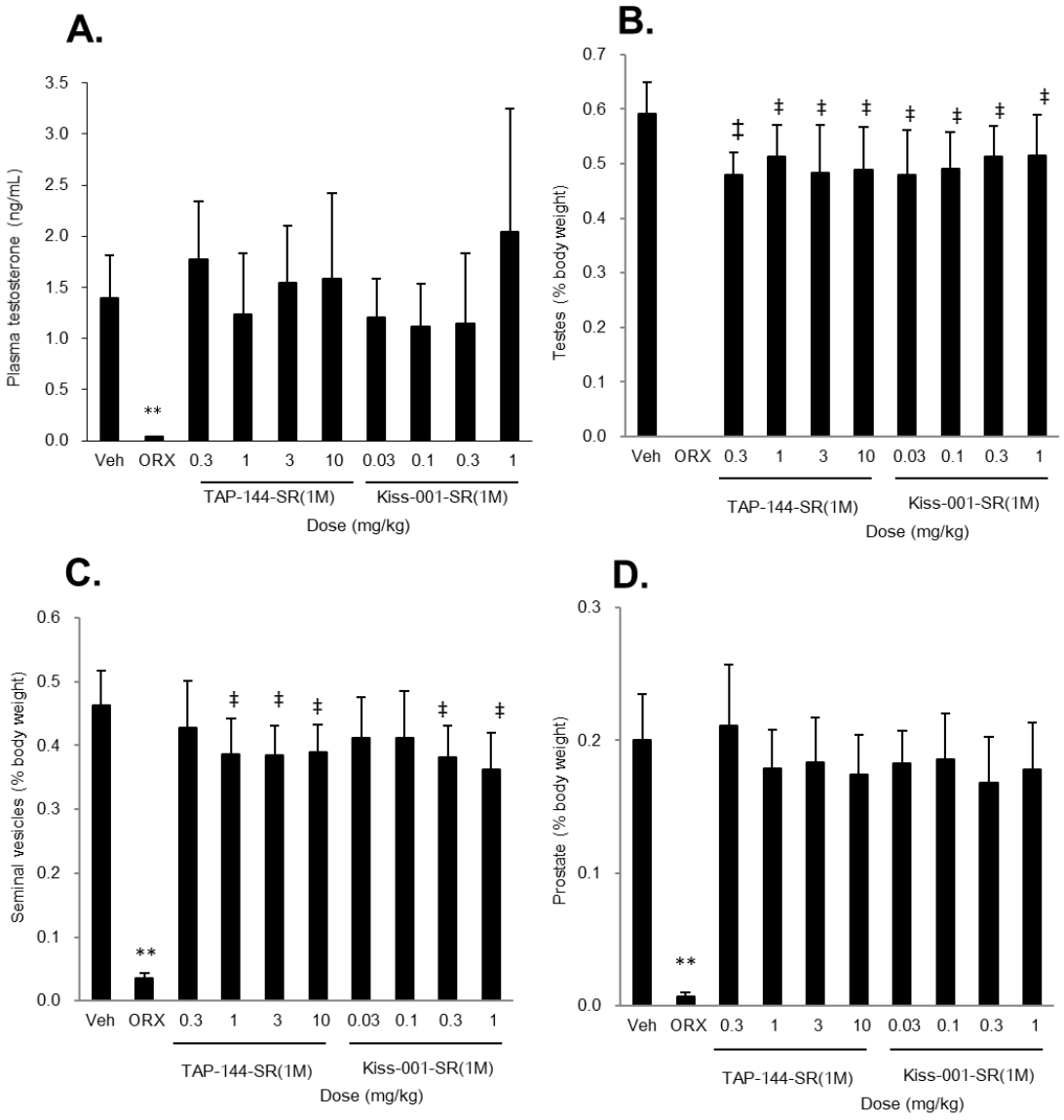

Fig. 16. Plasma testosterone levels and genital organ weights on day 84 after dosing of Kiss-001-SR(1M) or TAP-144-SR(1M) in adult male rats. Plasma testosterone levels (A) and weights of androgen-sensitive testes (B), seminal vesicles (C), and prostates (D) on day 84. Data represent means ± S.D. (n = 7). Veh: vehicle control. $^{**}P \leq 0.01$ vs. vehicle by the Wilcoxon rank sum test; $^{‡}P \leq 0.025$ vs. vehicle by the one-tailed Williams' test.

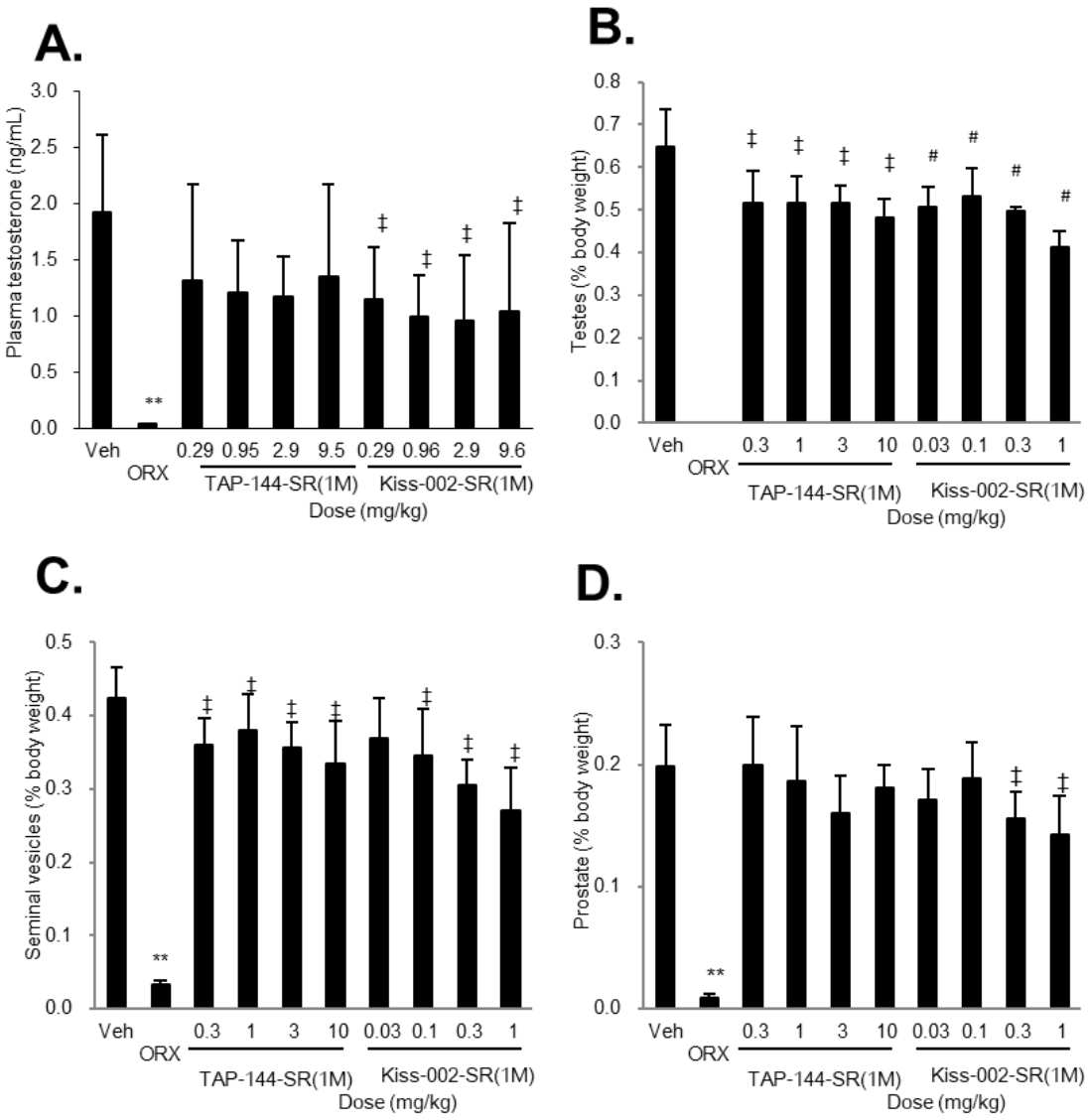

Fig. 17. Plasma testosterone levels and genital organ weights on day 84 after dosing of Kiss-002-SR(1M) or TAP-144-SR(1M) in adult male rats. (A–D) Plasma testosterone levels (A) and weights of androgen-sensitive testes (B), seminal vesicles (C), and prostates (D) on day 84. Data represent means ± S.D. (n = 7). Veh: vehicle control. $^{**}P \leq 0.01$ vs. vehicle by the Wilcoxon rank sum test; $^{\#}P \leq 0.025$ vs. vehicle by the one-tailed Shirley–Williams test; $^{‡}P \leq 0.025$ vs. vehicle by the one-tailed Williams' test.

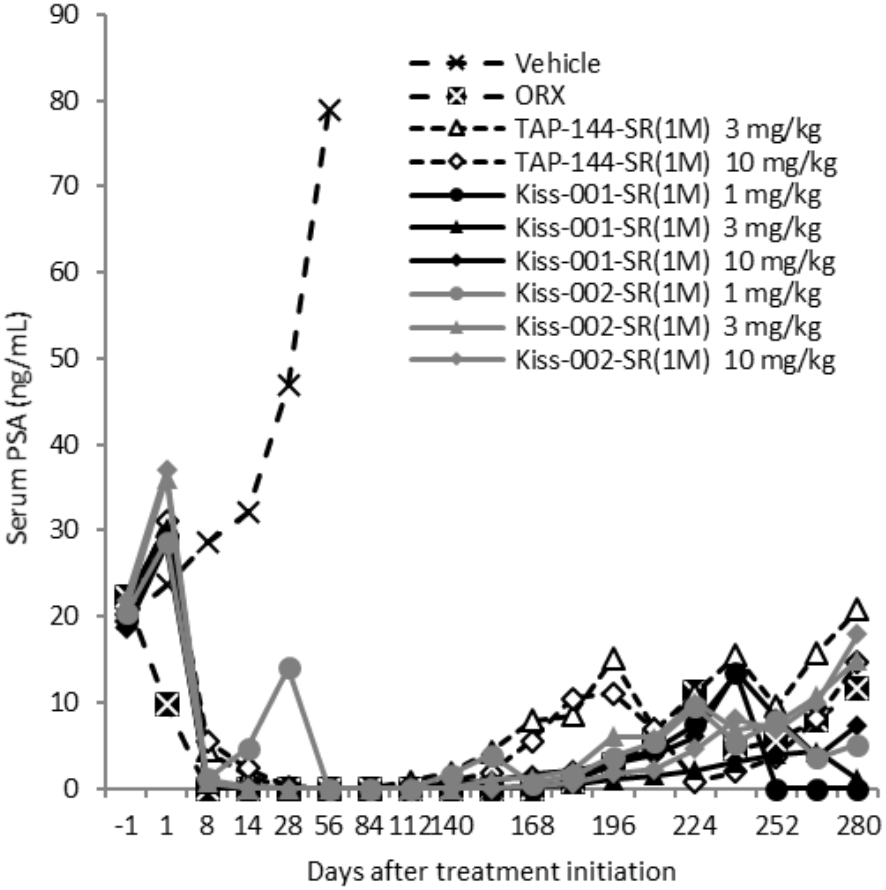

Fig. 18. Changes in plasma PSA levels by repeated monthly dosing of Kiss-001-SR(1M), Kiss-002-SR(1M), or TAP-144-SR(1M) in the JDCaP xenograft rat model. The changes in mean serum PSA levels are shown. All groups initially had 6 animals, but the numbers were decreased by deaths or euthanasia for PSA recurrence (PSA >20 ng/ml).

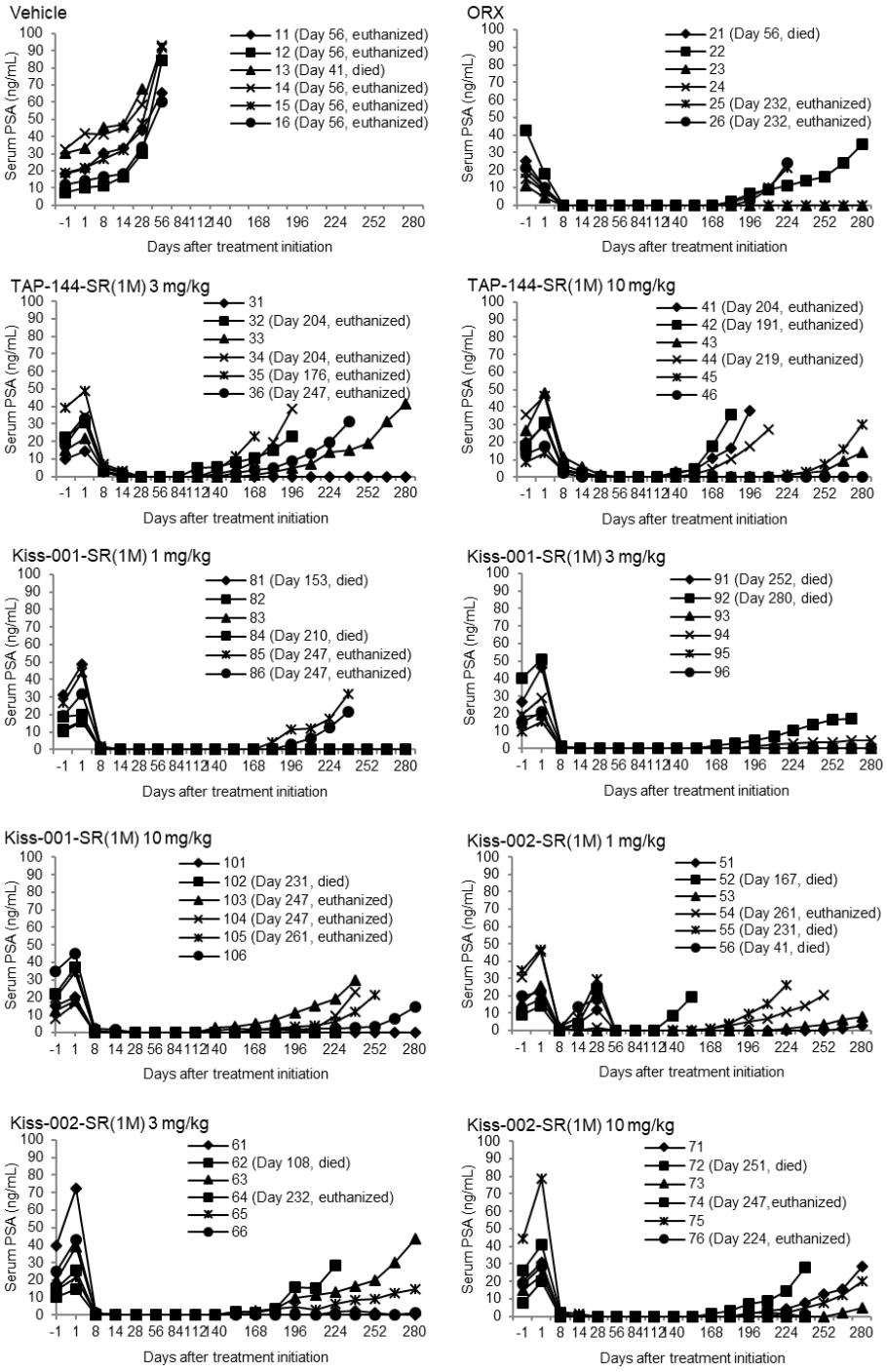

Fig. 19. Individual changes in plasma PSA levels by repeated monthly dosing of Kiss-001-SR(1M), Kiss-002-SR(1M), or TAP-144-SR(1M) in the JDCaP xenograft rat model. Numbers in legends indicate the individual animal number. All groups initially had 6 animals, but the numbers were decreased by deaths or euthanasia for PSA recurrence (PSA >20 ng/ml).

General discussion

Prostate cancer has still high prevalence rate in men worldwide and there is no curable treatment established. ADT is a gold standard therapy for advanced prostate cancer, however, eventually the cancer will be recurred by transforming to CRPC. Therefore, there is still an unmet medical need for a better ADT agent which can prolong or prevent the recurrence of prostate cancer. Thus, this study was aimed to develop a novel ADT agent which lead to better therapeutic outcome in the treatment of prostate cancer based on the better understanding of the mechanism regulating reproductive function including androgen synthesis in puberty.

In chapter 1, I firstly identified that at least two distinct hypothalamic regions, mPOA and ARC, are the action sites of estrogen for prepubertal restraint of GnRH/LH secretion in female rats. Estradiol microimplant in the mPOA or ARC, but not in the PVN or VMH, significantly suppressed LH secretion in prepubertal OVX rats. Interestingly, each suppressive effect on LH secretion of mPOA or ARC estradiol microimplant was relatively weaker than that of systemic estradiol replacement, suggesting that estrogen suppresses GnRH/LH secretion through a neural complex which consists of at least these two distinct estrogen responsive neurons located in the mPOA and ARC in prepubertal female rats.

I also identified that the responsiveness to estrogen in the mPOA is attenuated during puberty for the first time. On the other hand, there is no change in estrogen responsiveness in the ARC during pubertal development. Immunohistochemistry and gene expression analysis in my study revealed that the attenuation of estrogen responsiveness is not caused by the reduction of estrogen receptor expression because the

number of ERα-immunoreactive cells and the Esr1, Esr2 or Gpr30 gene expression in the mPOA and ARC were comparable between prepubertal and postpubertal period. Thus, it suggests that changes in neural inputs to GnRH neurons from a neural complex containing estrogen-responsive neurons located in the mPOA and ARC, rather than a change in the expression of ERs, would be involved in the attenuation of estrogen inhibitory action on GnRH/LH secretion observed in puberty.

Kisspeptin neuron is a plausible candidate for estrogen-responsive neuron in ARC involved in the mechanism regulating GnRH/LH secretion in puberty. ERα is expressed in ARC kisspeptin neurons (Kinoshita et al., 2005; Smith et al., 2005; Adachi et al., 2007) and ARC Kiss1 gene expression is nearly absent in the prepubertal period, however, increases toward postpubertal period (Takase et al., 2009). ARC Kiss1 expression was appeared by ovariectomy and was strongly suppressed by systemic estradiol replacement in prepubertal rats. In addition, my study demonstrated that LH secretion was evoked in response to multiple kisspeptin or GnRH subcutaneous injections in prepubertal OVX rats treated with systemic estradiol replacement. These findings indicate that estrogen-dependent prepubertal restraint of LH secretion would be due to the suppression of ARC kisspeptin and subsequent GnRH secretion.

With regard to the mPOA, there are a lot of estrogen responsive neurons such as GABAergic neuron (Flugge et al., 1986; Herbison AE, 1997), glutamatergic neuron (Eyigor et al., 2004), dopaminergic neuron (Yuri et al., 1994), and several peptidergic neurons (Herbison et al., 1992; Yuri et al., 1994; Bloch et al., 1992; Herbison et al., 1992; Axelson et al., 1992; Simerly et al., 1996). The involvement of these neurons in the mechanism regulating GnRH/LH secretion in puberty is still elusive. However, since there are several reports suggesting GABAergic inhibitory input is involved in the

mechanism regulating prepubertal GnRH/LH secretion (Ojeda et al., 2003, Feleder et al., 1999, Mitsushima et al., 1997, Feleder et al., 1996; Sacchi et al., 1998), I would like to raise one possibility that mPOA estrogen-responsive neurons exert an inhibitory input on ARC kisspeptin neuronal activity in prepubertal period. Further studies, e.g., projection of mPOA estrogen-responsive neurons into the ARC kisspeptin neurons, are required to understand this complicated neural network.

In chapter 2, I studied the application of kisspeptin analogs, Kiss-001 and Kiss-002, for the treatment of prostate cancer. I developed one-month sustained release depot of Kiss-001 and Kiss-002, Kiss-001-SR(1M) and Kiss-002-SR(1M), and determined their PK and PD profiles in intact male rats. As expected, both depots succeeded to maintain plasma Kiss-001F or Kiss-002F concentrations at certain levels for 4–5 weeks in intact male rats. Accompanying with these PK profiles, both Kiss-001-SR(1M) and Kiss-002-SR(1M) demonstrated more rapid and profound plasma testosterone suppression compared to GnRH analog TAP-144-SR(1M) which is currently used for the treatment of prostate cancer. Kiss-001-SR(1M) and Kiss-002-SR(1M) rapidly lowered plasma testosterone levels within 3 days after dosing and maintained plasma testosterone levels below LLOQ. on the other hand, the comparator TAP-144-SR(1M) showed continuous suppression of plasma testosterone levels in a dose-dependent manner as well, however, the initial transient increases in plasma testosterone level were longer than Kiss-001-SR(1M) and Kiss-002-SR(1M). In addition, the nadir value of the plasma testosterone level remained above the LLOQ even in the maximum dosing group (10 mg/kg).

My study in the JDCaP xenograft rat model demonstrated that both Kiss-001-SR(1M) and Kiss-002-SR(1M) rapidly achieved complete suppression of serum PSA levels within 8 days after treatment initiation, while TAP-144-SR(1M) required 4 weeks

to achieve the same condition. This finding suggests that Kiss-001-SR(1M) and Kiss-002-SR(1M) exert better PSA control during the initial phase of treatment than TAP-144-SR(1M) in patients with prostate cancer. All treatment groups eventually showed PSA recurrence, however, unexpectedly, Kiss-001-SR(1M) and Kiss-002-SR(1M) demonstrated tendency to delay the timing of PSA recurrence compared with TAP-144-SR(1M) in this animal model. Although the study size was too small to reach a concrete conclusion on the timing of PSA recurrence, these preliminary observations supports the hypothesis that better testosterone control with Kiss-001-SR(1M) and Kiss-002-SR(1M) could prolong the biochemical recurrence-free period in ADPC. Further studies are warranted to elucidate clinical significance of Kiss-001-SR(1M) and Kiss-002-SR(1M).

In conclusion, my study demonstrates that estrogen-responsive neurons located in the mPOA and ARC play key roles in pubertal change of GnRH/LH secretion in female rats and contributes better understanding on the mechanism regulating reproductive function in puberty. Also, my study indicates that kisspeptin analogs are applicable for the treatment of prostate cancer and Kiss-001-SR(1M) and Kiss-002-SR(1M) are the promising clinical candidates to be tested in patients with ADPC.

Acknowledgements

I am most grateful to Professors Kazuto Nakada, Kentaro Nakano and Tomoki Chiba and Associate Professor Yukihiko Tokunaga, University of Tsukuba, for their continuous guidance and valuable discussions through my doctoral program.

I also thank Professors Keiichirou Maeda and Hiroko Tsukamura, Associate Professor Yoshihisa Uenoyama, Nagoya University, for their clear guidance on my research.

I also thank Drs. Masami Kusaka, Tatsuya Watanabe, Taiji Asami, Hisanori Matsui and Kaori Ishikawa and many experts in Takeda Pharmaceutical Company Limited who dedicated to work in Metastin/Kisspeptin projects for their valuable discussions and suggestions.

I also express my gratitude to Drs. Sham Nikam and Hiroshi Nagabukuro, current supervisor at ARTham Therapeutics Inc, for their endorsement and support on my doctoral program..

Finally, I would like to appreciate my family who strongly supported my study in University of Tsukuba.

References

Adachi S, Yamada S, Takatsu Y, Matsui H, Kinoshita M, Takase K, Sugiura H, Ohtaki T, Matsumoto H, Uenoyama Y, Tsukamura H, Inoue K, Maeda KI. Involvement of anteroventral periventricular metastin/kisspeptin neurons in estrogen positive feedback action on luteinizing hormone release in female rats. J Reprod Dev 2007; 53: 367-378.

Andrews WW, Advis JP, Ojeda SR. The maturation of estradiol-negative feedback in female rats: evidence that the resetting of the hypothalamic "gonadostat" does not precede the first preovulatory surge of gonadotropins. Endocrinology 1981; 109: 2022-2031.

Andrews WW, Ojeda SR. A detailed analysis of the serum luteinizing hormone secretory profile in conscious, free-moving female rats during the time of puberty. Endocrinology 1981; 109: 2032-2039.

Asami T, Nishizawa N, Matsui H, Nishibori K, Ishibashi Y, Horikoshi Y, Nakayama M, Matsumoto S, Tarui N, Yamaguchi M, Matsumoto H, Ohtaki T, Kitada C. Design, synthesis, and biological evaluation of novel investigational nonapeptide KISS1R agonists with testosterone-suppressive activity J Med Chem. 2013: 56(21):8298-307

Axelson JF, Shannon W, Van Leeuwen FW. Immunocytochemical localization of estrogen receptors within neurotensin cells in the rostral preoptic area of the rat hypothalamus. Neurosci Lett 1992; 136: 5-9.Bloch GJ, Kurth SM, Akesson TR, Micevych PE. Estrogen-concentrating cells within cell groups of the medial preoptic area: sex differences and co-localization with galanin-immunoreactive cells. Brain Res 1992; 595: 301-308.

Cagampang FR, Maeda KI, Tsukamura H, Ohkura S, Ota K. Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH

release in ovariectomized rats. J Endocrinol 1991; 129: 321-328.

Chan YM, Broder-Fingert S, Wong KM, Seminara SB. Kisspeptin/Gpr54-independent gonadotrophin-releasing hormone activity in Kiss1 and Gpr54 mutant mice. J Neuroendocrinol 2009; 21: 1015-1023.

Chatani, F. (2006) Possible mechanism for testicular focal necrosis induced by hCG in rats. The Journal of toxicological sciences, 31, 291-303.

Chongthammakun S, Claypool LE, Terasawa E. Ovariectomy increases in vivo luteinizing hormone-releasing hormone release in pubertal, but not prepubertal, female rhesus monkeys. J Neuroendocrinol 1993; 5: 41-50.

Clarkson J, Herbison AE. Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. Endocrinology 2006; 147: 5817-5825.

Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 1999; 20: 358-417.

d'Anglemont de Tassigny X, Fagg LA, Dixon JP, Day K, Leitch HG, Hendrick AG, Zahn D, Franceschini I, Caraty A, Carlton MB, Aparicio SA, Colledge WH. Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. Proc Natl Acad Sci USA 2007; 104: 10714-10719.

de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc Natl Acad Sci USA 2003; 100: 10972-10976.

Dhillo WS, Chaudhri OB, Patterson M, Thompson EL, Murphy KG, Badman MK, McGowan BM, Amber V, Patel S, Ghatei MA, Bloom SR. Kisspeptin-54 stimulates the hypothalamic-pituitary gonadal axis in human males J Clin Endocrinol Metab. J Clin

Endocrinol Metab. 2005;90(12):6609-15

Dungan HM, Gottsch ML, Zeng H, Gragerov A, Bergmann JE, Vassilatis DK, Clifton DK, Steiner RA. The role of kisspeptin-GPR54 signaling in the tonic regulation and surge release of gonadotropin-releasing hormone/luteinizing hormone. J Neurosci 2007; 27: 12088-12095.

Edwards BK, Noone AM, Mariotto AB, Simard EP, Boscoe FP, Henley SJ, Jemal A, Cho H, Anderson RN, Kohler BA, Eheman CR, Ward EM. Annual Report to the Nation on the status of cancer, 1975-2010, featuring prevalence of comorbidity and impact on survival among persons with lung, colorectal, breast, or prostate cancer. Cancer. 2014;120(9):1290-314.

Estacio MA, Yamada S, Tsukamura H, Hirunagi K, Maeda K. Effect of fasting and immobilization stress on estrogen receptor immunoreactivity in the brain in ovariectomized female rats. Brain Res 1996; 717: 55-61.

Eyigor O, Lin W, Jennes L. Identification of neurones in the female rat hypothalamus that express oestrogen receptor-alpha and vesicular glutamate transporter-2. J Neuroendocrinol 2004; 16: 26-31.

Feleder C, Jarry H, Leonhardt S, Wuttke W, Moguilevsky JA. The GABAergic control of gonadotropin-releasing hormone secretion in male rats during sexual maturation involves effects on hypothalamic excitatory and inhibitory amino acid systems. Neuroendocrinology 1996; 64: 305-312.

Feleder C, Ginzburg M, Wuttke W, Moguilevsky JA, Arias P. GABAergic activation inhibits the hypothalamic-pituitary-ovaric axis and sexual development in the immature female rat. Associated changes in hypothalamic glutamatergic and taurinergic systems. Brain Res Dev Brain Res 1999; 116: 151-157.

Flugge G, Oertel WH, Wuttke W. Evidence for estrogen-receptive GABAergic neurons in the preoptic/anterior hypothalamic area of the rat brain. Neuroendocrinology 1986; 43: 1-5.

Foster DL, Yellon SM, Olster DH. Internal and external determinants of the timing of puberty in the female. J Reprod Fertil 1985; 75: 327-344.

Foster DL, Ebling FJP, Vannerson LA, Wood RI, Fenner DE. Regulation of puberty in the lamb: internal and external cues. In: Imura H, Shizume K, Yoshida S (eds.), Progress in Endocrinology. Amsterdam: Elsevier; 1988: 861-866.

George JT, Veldhuis JD, Roseweir AK, Newton CL, Faccenda E, Millar RP, Anderson RA. Kisspeptin-10 is a potent stimulator of LH and increases pulse frequency in men J Clin Endocrinol Metab. 2011;96(8):E1228-36.

Gottsch ML, Cunningham MJ, Smith JT, Popa SM, Acohido BV, Crowley WF, Seminara S, Clifton DK, Steiner RA. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology. 2004;145(9):4073-7.

Herbison AE, Theodosis DT. Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholecystokinin or luteinizing hormone-releasing hormone in the male and female rat. Neuroscience 1992; 50: 283-298.

Herbison AE, Theodosis DT. Immunocytochemical identification of oestrogen receptors in preoptic neurones containing calcitonin gene-related peptide in the male and female rat. Neuroendocrinology 1992; 56: 761-764.

Herbison AE. Estrogen regulation of GABA transmission in rat preoptic area. Brain Res Bull 1997; 44: 321-326.

Herbison AE, Pape JR. New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. Front Neuroendocrinol 2001; 22: 292-308.

Kimura T, Kiyota H, Nakata D, Masaki T, Kusaka M, Egawa S. A novel androgen-dependent prostate cancer xenograft model derived from skin metastasis of a Japanese patient Prostate. 2009; 69(15):1660-7.

Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, Yamada S, Inoue K, Ohtaki T, Matsumoto H, Maeda K. Involvement of central metastin in the regulation of preovulatory luteinizing hormone surge and estrous cyclicity in female rats. Endocrinology 2005; 146: 4431-4436.

Jeong-Hyung Lee, Mary E. Miele, Deana J. Hicks, Karen K. Phillips, Jeffery M. Trent, Bernard E. Weissman, Danny R. Welch. KiSS-1, a Novel Human Malignant Melanoma Metastasis-Suppressor Gene. J Natl Cancer Inst 1996;88: 1731-7

Kobayashi T, Sasaki S, Tomita N, Fukui S, Nakayama M, Kiba A, Kusaka M, Matsumoto S, Yamaguchi M, Itoh F, Baba A. 2-acylamino-4,6-diphenylpyridine derivatives as novel GPR54 antagonists with good brain exposure and in vivo efficacy for plasma LH level in male rats. Bioorg Med Chem. 2010;18(14):5157-71.

Lapatto R, Pallais JC, Zhang D, Chan YM, Mahan A, Cerrato F, Le WW, Hoffman GE, Seminara SB. Kiss1-/- mice exhibit more variable hypogonadism than Gpr54-/- mice. Endocrinology 2007; 148: 4927-4936.

Lomniczi A, Loche A, Castellano JM, Ronnekleiv OK, Bosch M, Kaidar G, Knoll JG, Wright H, Pfeifer GP, Ojeda SR. Epigenetic control of female puberty. Nat Neurosci 2013; 16: 281-289.

MacLean DB, Matsui H, Suri A, Neuwirth R, Colombel M. Sustained Exposure to the Investigational Kisspeptin Analog, TAK-448, Down-Regulates Testosterone into the Castration Range in Healthy Males and in Patients With Prostate Cancer: Results From Two Phase 1 Studies J Clin Endocrinol Metab. 2014; 99(8):E1445-53.

Matsui H, Masaki T, Akinaga Y, Kiba A, Takatsu Y, Nakata D, Tanaka A, Ban J, Matsumoto S, Kumano S, Suzuki A, Ikeda Y, Yamaguchi M, Watanabe T, Ohtaki T, Kusaka M. Pharmacologic profiles of investigational kisspeptin/metastin analogues, TAK-448 and TAK-683, in adult male rats in comparison to the GnRH analogue leuprolide Eur J Pharmacol. 2014; 15;735:77-85.

Merriam GR, Wachter KW. Algorithms for the study of episodic hormone secretion. Am J Physiol 1982; 243: E310-318.

Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, Thresher RR, Malinge I, Lomet D, Carlton MB, Colledge WH, Caraty A, Aparicio SA. Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. Proc Natl Acad Sci USA 2005; 102: 1761-1766.

Mitsushima D, Kimura F. The maturation of GABA(A) receptor-mediated control of luteinizing hormone secretion in immature male rats. Brain Res 1997; 748: 258-262.

Mohler JL, Antonarakis ES, Armstrong AJ, D'Amico AV, Davis BJ, Dorff T, Eastham JA, Enke CA, Farrington TA, Higano CS, Horwitz E, Hurwitz M, Ippolito JE, Kane CJ, Kuettel MR, Lang JM, McKenney J, Netto G, Penson DF, Plimack ER, Pow-Sang JM, Pugh TJ, Richey S, Roach M, Rosenfeld S, Schaeffer E, Shabsigh A, Small EJ, Spratt DE, Srinivas S, Tward J, Shead DA and Freedman-Cass DA. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®). JNCCN 2019; 17(5): 479-505

Nagatani S, Tsukamura H, Maeda K. Estrogen feedback needed at the paraventricular nucleus or A2 to suppress pulsatile luteinizing hormone release in fasting female rats. Endocrinology 1994; 135: 870-875.

Navarro VM, Castellano JM, Fernandez-Fernandez R, Barreiro ML, Roa J, Sanchez-Criado JE, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. Developmental and

hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 2004; 145: 4565-4574.

Nishizawa N, Takatsu Y, Kumano S, Kiba A, Ban J, Tsutsumi S, Matsui H, Matsumoto SI, Yamaguchi M, Ikeda Y, Kusaka M, Ohtaki T, Itoh F, Asami T. Design and Synthesis of an Investigational Nonapeptide KISS1 Receptor (KISS1R) Agonist, Ac-d-Tyr-Hydroxyproline (Hyp)-Asn-Thr-Phe-azaGly-Leu-Arg(Me)-Trp-NH2 (TAK-448), with Highly Potent Testosterone-Suppressive Activity and Excellent Water Solubility. J Med Chem. 2016;59(19):8804-8811.

Ohkura S, Takase K, Matsuyama S, Mogi K, Ichimaru T, Wakabayashi Y, Uenoyama Y, Mori Y, Steiner RA, Tsukamura H, Maeda KI, Okamura H. Gonadotrophin-releasing hormone pulse generator activity in the hypothalamus of the goat. J Neuroendocrinol. 2009;21(10):813-21.

Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, Asada M, Yamada T, Suenaga M, Kitada C, Usuki S, Kurokawa T, Onda H, Nishimura O, Fujino M. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature. 2001;411(6837):613-7.

Ojeda SR, Prevot V, Heger S, Lomniczi A, Dziedzic B, Mungenast A. The neurobiology of female puberty. Horm Res 2003; 60 Suppl 3: 15-20.

Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. San Diego, Academic Press: 2007.

Pohl CR, deRidder CM, Plant TM. Gonadal and nongonadal mechanisms contribute to the prepubertal hiatus in gonadotropin secretion in the female rhesus monkey (Macaca

mulatta). J Clin Endocrinol Metab 1995; 80: 2094-2101.

Prashanth Rawla. Epidemiology of Prostate Cancer. World J Oncol. 2019; 10(2): 63–89.

Ramaswamy S1, Seminara SB, Pohl CR, DiPietro MJ, Crowley WF Jr, Plant TM. Effect of continuous intravenous administration of human metastin 45-54 on the neuroendocrine activity of the hypothalamic-pituitary-testicular axis in the adult male rhesus monkey (Macaca mulatta). Endocrinology. 2007;148(7):3364-70

Rajagopal S, Shenoy SK. GPCR desensitization: Acute and prolonged phases. Cell Signal. 2018; 41:9-16

Roseweir AK, Millar RP. Kisspeptin antagonists. Adv Exp Med Biol. 2013; 784:159-86.

Scacchi P, Carbone S, Szwarcfarb B, Rondina D, Wuttke W, Moguilevsky JA. Interactions between GABAergic and serotoninergic systems with excitatory amino acid neurotransmission in the hypothalamic control of gonadotropin secretion in prepubertal female rats. Brain Res Dev Brain Res 1998; 105: 51-58.

Scott G, Ahmad I, Howard K, MacLean D, Oliva C, Warrington S, Wilbraham D, Worthington P. Double-blind, randomized, placebo-controlled study of safety, tolerability, pharmacokinetics and pharmacodynamics of TAK-683, an investigational metastin analogue in healthy men. Br J Clin Pharmacol. 2013;75(2):381-91.

Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Jr., Shagoury JK, Bo-Abbas Y, Kuohung W, Schwinof KM, Hendrick AG, Zahn D, Dixon J, Kaiser UB, Slaugenhaupt SA, Gusella JF, O'Rahilly S, Carlton MB, Crowley WF, Jr., Aparicio SA, Colledge WH. The GPR54 gene as a regulator of puberty. N Engl J Med 2003; 349: 1614-1627.

Seminara SB, Dipietro MJ, Ramaswamy S, Crowley WF Jr, Plant TM. Continuous human metastin 45-54 infusion desensitizes G protein-coupled receptor 54-induced

gonadotropin-releasing hormone release monitored indirectly in the juvenile male Rhesus monkey (Macaca mulatta): a finding with therapeutic implications. Endocrinology. 2006;147(5):2122-6.

Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. Proc Natl Acad Sci U S A. 2005;102(6):2129-34.

Shivers BD, Harlan RE, Morrell JI, Pfaff DW. Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature 1983; 304: 345-347.

Sisk CL, Richardson HN, Chappell PE, Levine JE. In vivo gonadotropin-releasing hormone secretion in female rats during peripubertal development and on proestrus. Endocrinology 2001; 142: 2929-2936.

Simerly RB, Young BJ, Carr AM. Co-expression of steroid hormone receptors in opioid peptide-containing neurons correlates with patterns of gene expression during the estrous cycle. Brain Res Mol Brain Res 1996; 40: 275-284.

Smith JT, Cunningham MJ, Rissman EF, Clifton DK, Steiner RA. Regulation of Kiss1 gene expression in the brain of the female mouse. Endocrinology 2005; 146: 3686-3692.

Takase K, Uenoyama Y, Inoue N, Matsui H, Yamada S, Shimizu M, Homma T, Tomikawa J, Kanda S, Matsumoto H, Oka Y, Tsukamura H, Maeda KI. Possible role of oestrogen in pubertal increase of Kiss1/kisspeptin expression in discrete hypothalamic areas of female rats. J Neuroendocrinol, 2009; 21: 527-537.

Tanaka A, Matsui H, Asami T, Nishizawa N, Kitada C, Ohtaki T, MacLean D, Kusaka M. 656 Suppression of testosterone release by chronic administration of investigational novel metastin analogues in male dogs and monkeys, and in healthy male volunteers. European Journal of Cancer Supplements. 2010; 8(7)205

Thompson EL, Murphy KG, Patterson M, Bewick GA, Stamp GW, Curtis AE, Cooke JH, Jethwa PH, Todd JF, Ghatei MA, Bloom SR. Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats Am J Physiol Endocrinol Metab. Am J Physiol Endocrinol Metab. 2006;291(5):E1074-82.

Thompson EL, Patterson M, Murphy KG, Smith KL, Dhillo WS, Todd JF, Ghatei MA, Bloom SR. Central and peripheral administration of kisspeptin-10 stimulates the hypothalamic-pituitary-gonadal axis J Neuroendocrinol. J Neuroendocrinol. 2004;16(10):850-8.

Toguchi H, Ogawa Y, Okada H, Yamamoto M. [Once-a-month injectable microcapsules of leuprorelin acetate] Yakugaku Zasshi. 1991;111(8):397-409.

Tomikawa J, Uenoyama Y, Ozawa M, Fukanuma T, Takase K, Goto T, Abe H, Ieda N, Minabe S, Deura C, Inoue N, Sanbo M, Tomita K, Hirabayashi M, Tanaka S, Imamura T, Okamura H, Maeda KI, Tsukamura H. Epigenetic regulation of Kiss1 gene expression mediating estrogen-positive feedback action in the mouse brain. Proc Natl Acad Sci U S A 2012; 109: E1294-1301.

Topaloglu AK, Tello JA, Kotan LD, Ozbek MN, Yilmaz MB, Erdogan S, Gurbuz F, Temiz F, Millar RP, Yuksel B. Inactivating KISS1 mutation and hypogonadotropic hypogonadism. N Engl J Med 2012; 366: 629-635.

Watanabe G, Terasawa E. In vivo release of luteinizing hormone releasing hormone increases with puberty in the female rhesus monkey. Endocrinology 1989; 125: 92-99.

Yamada S, Uenoyama Y, Maeda K, Tsukamura H. Role of noradrenergic receptors in the bed nucleus of the stria terminalis in regulating pulsatile luteinizing hormone secretion in female rats. J Reprod Dev 2006; 52: 115-121.

Yuri K, Kawata M. Estrogen receptor-immunoreactive neurons contain calcitonin gene-

related peptide, methionine-enkephalin or tyrosine hydroxylase in the female rat preoptic area. Neurosci Res 1994; 21: 135-141.